Interactions between the cholinergic and dopaminergic systems during the production of ultrasonic vocalizations in rats

Michael J. D. Silkstone, B. Sc.

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Abstract

Rats produce ultrasonic vocalizations that can be categorized into two types of ultrasonic calls based on their sonographic structure. One group contains 22-kHz ultrasonic vocalization (USVs), characterized by relatively constant (flat) frequency with peak frequency ranging from 19 to 28-kHz, and a call duration ranging between 100 – 3000 ms. These vocalization can be induced by cholinomimetic agents injected into the ascending mesolimbic cholinergic system that terminates in the anterior hypothalamic-preoptic area (AH-MPO) and lateral septum (LS). The other group of USVs contains 50-kHz USVs, characterized by high peak frequency, ranging from 39 to 90-kHz, short duration ranging from 10-90 ms, and varying frequency and complex sonographic morphology. These vocalizations can be induced by dopaminergic agents injected into the nucleus accumbens, the target area for the mesolimbic dopaminergic system. 22-kHz USVs are emitted in situations that are highly aversive, such as proximity of a predator or anticipation of a foot shock, while 50 kHz USVs are emitted in rewarding and appetitive situations, such as juvenile play behaviour or anticipation of rewarding electrical brain stimulation. The activities of these two mesolimbic systems were postulated to be antagonistic to each other. The current thesis is focused on the interaction of these systems indexed by emission of relevant USVs. It was hypothesized that emission of 22 kHz USVs will be antagonized by prior activation of the dopaminergic system while emission of 50 kHz will be antagonized by prior activation of the cholinergic system. It was found that injection of apomorphine into the shell of the nucleus accumbens significantly decreased the number of carbachol-induced 22 kHz USVs from both AH-MPO and LS. Injection of carbachol into the LS significantly decreased the number of apomorphine-induced 50 kHz USVs from the shell of the nucleus accumbens. The results of the study supported the main hypotheses that the mesolimbic dopaminergic and cholinergic systems function in antagonism to each other.

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AH-MPO = Anterior Hypothalamic – Medial Preoptic Area

BG = Basal Ganglia

c-fos-IR = *c-fos*- immunoreactivity

DDC = Dorsal Diencephalic Conduction System

DOPAC = 3, 4-dihydroxyphenylacetic acid

DRG = Dorsal Respiratory Group

FM = Frequency Modulated 50-kHz USV

fr = fasciculus retroflexus

GAD = Glutamic acid decarboxylase

IEG = Immediate Early Gene

IPN = Interpeduncular Nucleus

HVA = Homovanillic Acid

LH = Lateral Habenula

LS = Lateral Septum

LV = Lateral Ventricle

LTDg = Laterodorsal Tegmental Nucleus

MCVS = Medial cholinoceptive vocalization strip

MFB = Medial Forebrain Bundle

PAG = Periaqueductal Gray

PPT = Pedunculopontine Tegmental Nucleus

sNa = shell of the nucleus accumbens

TTX = Tetrodotoxin

USV = Ultrasonic Vocalization

VRG = Ventral Respiratory Group

VTA = Ventral Tegmental Area

Introduction

Emotions

Emotions are internal states that allow an animal to regulate and maintain motivational behaviours when interacting within the environment (Griffiths, 2001; Rolls, 2001). Emotions help preserve arousal and motivational states that are involved in the procurement of food, water and essential nutrients that help maintain an internal homeostasis. Emotions also help preserve complex motivational states and help animals navigate complex social interactions within a group or a colony (Rolls, 2001). Regulating an animal's interaction within the social environment is dependent on the ability to communicate a variety of different emotionally-related signals to group members. The method of communication has evolved concurrently with the complexity of the social group allowing for the optimization of signal transmission.

Communication by animals involves two components. The first component in the act of communicating involves the propagation of a signal which has been converted into a sign. The sign that has been encrypted contains the semiotic content that is associated with the "message", which has to be decoded by the brain of the receiver (Brudzynski, 2005). Once the receiver has decoded and interpreted the sign the semiotic content has the ability to alter the behaviour or physiological processes of the receiver. An example of such a relationship between the sender and the receiver is demonstrated in a number of mammalian species. For example, Vervet monkeys have evolved different signs that communicate information signaling the location of aerial predators and different signs that communicate information signaling terrestrial predators, such as leopards and snakes (Seyfarth et al, 1980). The different alarm calls initiate different behaviours in monkeys. Terrestrial alarm calls alerting group members to a snake or leopard cause the monkeys to run up trees, while aerial alarm calls cause the monkeys to run into dense

brush and/or look to the sky (Seryfarth et al, 1980). Similarly, salamanders and fishes have sophisticated chemical signals to signal species-typical mating cues (Ptacek, 2000) to attract mates. Although many forms of communication exist between the signaler and the receiver, one of the most prolific forms of communication in mammals is by means of vocalization (Brudzynski, 2005; 2007). Vocalization (or acoustic communication) offers distinct advantages over other forms of communication like chemical signaling or visual signaling. Vocal communication is not dependent upon light, is not dependent upon proximity of conspecifics, the pitch, duration and frequency of vocal communication can encode robust messages and vocal communication can be terminated immediately.

Vocal Communication in mammals

Vocal communication is produced by pressurized air released through vibrating focal folds in the larynx. The power of the produced sound thus depends on the lung pressure, the aperture of the vocal folds and the frequency in which the vocal folds vibrate (Fletcher, 2010). As the pressure wave propagates away from the source, it is subjected to multiple environmental conditions such as energy absorption by the air, ground impedance and absorption/reflection by various environmental objects (Fletcher, 2010). This makes the source of the vocalization difficult to localize by predators and allows signs to be communicated without being dependent upon visibility between the sender and the receiver. Vocal communication also allows for the transmission of information to be independent of the availability of light. The ability to communicate in the absence of being visible is an adaptive advantage in rodents who are prey for a variety of different carnivores that hunt in the day and night (Brudzynski, 2005; Brudzynski and Fletcher, 2010).

Communication in rats

Both audible vocalization and ultrasonic vocalization (USVs) in rats communicate semiotic information that contains several adaptive values. Audible vocalizations refer to vocalizations that are emitted by rats that have a frequency range of 2-4 kHz (Brudzynski, 2007). Audible vocalizations in rats can be emitted under situation of physical pain (Borszcz, 2006) or under situations where physical pain is anticipated. Audible vocalizations in rats can also be emitted in response to approaching predators signaling that the rat is ready for defensive attacks (Litvin et al. 2007).

Vocalizations in rats that have a frequency range from 19 to 100- kHz are called ultrasonic vocalizations (USVs). USVs can be quantified by their peak frequency which is measured in kHz, their duration which is measured in milliseconds (ms), their bandwidth which is the difference between the maximum and minimum frequency of the call, and by their structural morphology (Brudzynski, 2007). USVs emitted by rats ranging from 19- to 100-kHz are classified into two different forms of USVs. One type of USVs is termed defensive-based 22-kHz USVs and is quantified as having a peak frequency between 19- to 32-kHz, a duration that ranges from 100 – 3000 ms and a consistently flat bandwidth (Bihari et al, 2003). The second form of USVs is categorized as 50-kHz USVs and they are quantified by their peak frequencies ranging from 45- to 90-kHz, there relatively short durations which range from 10 – 90 ms, and their highly variable bandwidth (Burgdorf, 2000; 2001). The production of USVs that have parameters that fall within the range of 22-kHz are able to index the signalers negative-emotional state since these vocalizations are produced in punishing circumstances. The production of USVs that have parameters which fall within the range of 50-kHz USVs are able to index the signalers positive-emotional state since these vocalizations are producing during positive pro-social

circumstances. Both forms of USVs have adaptive values and are important for the survival of the animal and thus, remain mutually antagonoistic to each other in order to preserve the sign being communicated.

Evidence that supports the idea that 22-kHz USVs are defensive alarm calls that index a negative-emotional state in the signaler

Development of a defensive system is critical to ensure the survival of prey species. The outbred Long-Evans rat, which belongs to *Rattus norvegicus*, has developed that ability to emit 22-kHz USVs as a defensive alarm call. The idea that 22-kHz USVs represent an alarm call or a defensive vocalization was first championed by a seminal publication by Blanchard and Blanchard (1991). The paper described the behaviour of rats after a predator was introduced into their immediate environment, which induced the emission of 22-kHz and subsequent immobility in the rats living within a visible burrow system. These USVs were seen long after the cat was removed and were argued to act as a method of transmitting the proximity of a predator to the colony. Evidence that further supports the conclusion that 22-kHz USVs are produced in aversive circumstances are demonstrated in experiments that utilize playback recordings, social transmission of fear and *c-fos*-immunoreactivity in response to 22-kHz USVs (Wöhr and Schwarting 2011).

A method for testing the ability of 22-kHz USVs to alter the behaviour of a receiver is to play the ultrasounds through a specialized speaker system and quantify the locomotor activity and behaviour of the receiver. If the animal executes motor patterns consistent with fear or anxiety it will become immobile or execute hopping and jumping motor patterns that are akin to escape behaviours (Brandão et al., 2008). Brudzynski and Chiu (1995) induced and recorded 22-kHz USVs and subsequently played the aversive 22-kHz back to rats for several seconds. During

the playback of the 22-kHz USVs recording, the locomotion of the rats did not change, but once the ultrasound was terminated the rats showed significant decreases in locomotion (Brudzynski and Chiu, 1995). Although these studies used recording of a tactile-induced 22-kHz USVs similar results are reported in studies using pair-housed rats to socially transmitted 22-kHz USVs.

Kim and colleagues (2010) reported results that demonstrated the defensive behavioural changes that occur in the receiver of 22-kHz USVs during fear conditioning and pair-testing. Using a fear conditioning chamber, Kim and colleagues (2010) conditioned one animal to a pairing of a tone and foot shock. After the animal had acquired conditioned fear, which was assessed by freezing and the production of 22-kHz USVs in the chamber, the conditioned animal was placed with a naïve cage mate in a novel chamber. During the initial phases of the pairing, both animals showed normal exploratory behaviour but once the conditioned tone was presented both animals showed freezing behaviour and production of 22-kHz USVs for the entire 8 minute testing session (Kim et al, 2010). These results support the original argument presented by Blanchard and Blanchard (1991) that 22-kHz USVs induce defensive behaviours in the receivers.

Further support to the idea that aversive semiotic content is carried by 22-kHz USVs to the receivers is highlighted by *c-fos* immunoreactivity experiments. Using playback of naturally induced 22-kHz USVs Sadananda and colleagues (2007) reported differential Fos-like immunoreactivity in key brain regions that are associated with fear and anxiety. Fos-like immunoreactivity was significantly increased in regions of the midbrain and forebrain of the rat, and included structures such as the lateral amygdala and the periaquductal gray (PAG; Sandananda et al, 2007). Electrophysiological analysis has reported that all the above-mentioned nuclei are involved in fear conditioning and defensive behavioural responses.

Using an auditory conditioning paradigm that was paired with foot-shocks, Quirk and colleagues reported that almost a quarter of the neurons within the lateral amygdala showed increases in firing rates compared to controls in response to a conditioned tone (Quirk et al., 1995). In agreement with the idea that the lateral amygdala is involved in acoustic emotional states, lesions placed within the lateral amygdala disrupt emotional conditioning (LeDoux et al., 1990). It is also interesting to note that the lateral amygdala receives dense projections from the acoustic thalamus (LeDoux et al., 1990). This would position the lateral amygdala to process 22-kHz USVs and send the information to relevant brain structures such as the PAG to execute a variety of behavioural responses such as freezing, changes in autonomic activity, execution of defensive behaviour or analgesia (Bessen et al., 1992; Bandler and Shipley, 2003).

The production of 22-kHz USVs is not limited to the approach of a predator or in anticipation of aversive stimuli but can be produced under a number of different socially aversive situations. The productions of 22-kHz USVs have been observed during social defeat after the introduction of an intruder male into a "resident" male's cage (Burgdorf et al, 2008). Aggression was indexed by the number of bites (>3) the "intruder" received and vocal analysis showed a large increase in the defeated animal to produce 22-kHz USVs (Burgdorf et al, 2008). Aversive 22-kHz USVs have also been recorded by investigators during aversive handling (Brudzynski and Ociepa, 1992), application of a jet of air to the head of the rat (Knapp and Pohorecky, 1995), when the rat is acoustically startled (Kaltwasser, 1990), when the rat anticipates the application of a foot-shock (De Vry et al, 1993), or during auditory or visual fear conditioning (Jelen et al., 2003).

Pharmacological evidence also supports the notion that 22-kHz USVs index a negative emotional state of the rat. Anxiolytics are drugs that decrease the self-indexed report anxiety in

the human population while anxiogenics are drugs that can increase the self-indexed report of anxiety within the human population. Drugs that are typically classified as anixolytics or anti-depressants including diazepam, chlordiazepoxide, meprobamate, pentobarbital, and gepirone, significantly decrease the number of emitted 22-kHz USVs in conditioned fear paradigms (De Vry et al, 1993). In contrast anxiogenics such as PTZ and FG-7142 have the ability to increase the production of 22-kHz USVs in conditioned fear paradigms over controls (Jelen et al, 2003).

Neural systems associated with the initiation of defensive behaviours and the 22-kHz USVs

Behaviours are external manifestations of internal processes occurring throughout the nervous system. If 22-kHz USVs are components of a large "defensive" mechanism of the rat then there should be discrete nuclei localized within the rat brain that are predisposed to the production of defensive behaviors, and subsequently the production of 22-kHz USVs.

Investigations of the brain nuclei that are responsible for assembling defensive behaviours in the animal began with decorticated dogs and cats. These decorticated animals would show extreme emotional reactivity to benign sensory stimuli like pinching the tail of the animal, a fly landing on the nose, or being removed from their home cage (Bard, 1929; 1934). To explain these emotional disturbances in the decorticated animal subjects, Cannon put forth a proposed diencephalic mechanism of emotion, focusing on the thalamus (Cannon & Newton, 1929). The conceptual frame work of Cannon's model was the processes of disinhibition of the thalamus by specific patterns of cortical activity (Cannon, 1927). The pattern of disinhibition from the thalamus would produce different emotional or affective states. This information would then be transmitted to the motor system to initiate the expression of the current emotion (Cannon, 1927). The investigation of defensive circuits localized within the brain was furthered by Bard.

Bard was able to show that the neural regions responsible for organizing defensive behaviours in cats were not localized within the thalamus but rather were localized within the hypothalamus. Bard decorticated cats while at the same time systematically destroyed specific parts of the forebrain or midbrain in the cat. Decorticated cats that also had their thalamus damaged were still able to produce stereotypical rage responses which included protrusion of claws, lashing of the tails, rapid panting, snarling, spitting, piloerection, and sweating from the pads of the feet. Animal subjects that were decorticated and also had damage to the hypothalamus or damage extending into the brain stem failed to produce any behavioural reactions to benign stimulation (Bard, 1929). Bard ended up correctly concluding that the hypothalamus was responsible for organizing defensive behaviours in cats as long as the hypothalamus remained intact with the brainstem.

The hypothesis that Bard derived about the hypothalamus being able to coordinate defensive behaviours was later supported by Hess. Hess used electrical stimulation of brain structures located in the forebrain and midbrain of the cat that were involved in the organization and expression of defensive behaviours. Hess's investigation in cats showed circumscribed regions in the hypothalamus that were responsible for affective defense reactions and included the medial preoptic area and perifornical area (Hess and Akert, 1955). The defensive reaction in cats that Hess was able to induce by electrically stimulating the hypothalamus was akin to the defensive reactions described by Bard and Cannon in their decorticated cats. Although the hypothalamus was correctly identified as being necessary for the production of defensive behaviours, electrical stimulation made it impossible to identify the different neurochemicals or specific neural pathways that are responsible for initiating such complex defensive behaviour.

The technique of electrical stimulation was very good at exciting nerve cells but the technique was non-specific and could excite fibers of passage leading to the activation of undesired nuclei. This led to problems of correlating behaviours that were organized by brain nuclei. The problem of electrical stimulation was later replaced by the technique of intracerebral injections. This technique allows for chemicals to be directly injected into nuclei of the subject's brain. Since fibers of passage do not respond to transmitters, they are not influenced by the injected chemicals. The method of intracerebral injections was used to shed light on the neurochemicals that were involved in the initiation of defensive response in cats.

The method of intracerebral injections was able to provide evidence that implicated acetylcholine in the initiation of defensive reactions in the cat when injected into the medial and anterior parts of the hypothalamus (Brudzynski, 1981a, b). Injection of carbachol, a cholinergic agonist specific for muscarinic receptors, into the anterior and medial parts of the hypothalamus (AH-MPO) were able to produce somatic and behavioral components of defensive responses that were consistent with defensive behaviors reported by Hess (1955), Cannon (1927) and Bard (1928). This provided evidence that the initiation of defensive behaviour in the cat was somehow dependent upon acetylcholine being released from the terminal bouton, diffusing across the synaptic cleft, and binding to muscarinic acetylcholine receptors along the midbrain-forebrain continuum.

In the cat cholinergic innervation of the anterior and medial parts of the hypothalamus originates from the laterodorsal tegmental nucleus (LTDg), a group of cholinergic cell bodies located within the mesopontine tegmentum (Kimura et al, 1981). These cholinergic cell bodies project and innervate parts of the hypothalamus and other subcortical structures such as the lateral septum (LS). This cholinergic anatomical projection system that innervates parts of the

hypothalamus responsible for organization and initiation of defensive behaviours in cats is also found in Long-Evan rats (Paxinos and Watson, 1986; Chiba & Murata, 1985; Cornwall et al, 1990).

In rats, the LTDg has a similar trajectory to cats and also has the ability to initiate defensive displays (Brudzynski,and Barnabi, 1996). Stimulation of the LTDg in the rat by direct injection of the excitatory amino acid glutamate can cause robust initiation of species-typical 22-kHz USVs alarm calls (Brudzynski and Barnabi, 1996). These defensive 22-kHz USVs can be significantly reduced if the anterior hypothalamic – medial preoptic region (AH-MPO) of the rat is pretreated with the muscarinic antagonist scopolamine (Brudzynski and Barnabi, 1996). The parameters of the 22-kHz USVs that are produced under pharmacological initiation do not significantly differ from defensive alarm calls that are produced by a rat in response to a predator (Brudzynski, 2007).

The production of defensive 22-kHz USVs in rats can also be initiated by carbachol being injected into extra-hypothalamic brain regions localized within the basal forebrain. Injection of carbachol into the lateral septum (LS) can initiate 22-kHz USVs (Bihari et al, 2003). Similarly, 22-kHz USVs induced by glutamate injections into the LTDg can be decreased by pretreatment of the LS with atropine or scopolamine, both of which are muscarinic antagonists (Bihari, et al, 2003). Both the LS and the AH-MPO are nuclei that belong to a larger system of brain structures arranged along the midbrain-forebrain continuum termed the medial ascending cholinoceptive vocalization strip (Brudzynski, 2007). Brain structures that are localized within the medially ascending strip are able to produce 22-kHz USVs in response to injection of carbachol or are able to decrease glutamate induced 22-kHz USVs from the LTDg by prior application of muscarinic antagonists.

The ascending cholinoceptive vocalization strip was defined as a longitudinally arranged strip of tissue that extends from the mesencephalon to the basal forebrain that is involved in the production of defensive alarm vocalization (Brudzynski 2007; 2010; 2011). Although this system was first investigated in the cat (Brudzynski, 1981a, b) it has become extensively investigated within the rat. The system functions in an ascending manner whereby excitation of the LTDg causes the subsequent release of acetylcholine in the AH-MPO, LS and other target sites to initiate defensive behaviours, such as the production of 22-kHz USVs.

Conclusions from the production of 22-kHz USVs in rats

From the described behavioural and pharmacological evidence it is apparent that the production of 22-kHz USVs represents a defensive vocal behaviour in the rat. This behaviour can be initiated in behavioural experiments involving conditioned fear, or negative social interactions. Aversive 22-kHz USVs can also be induced pharmacologically by carbachol given into synapses along the ascending cholinoceptive vocalization strip. In rats, as in cats, one of the key neurotransmitter receptor that is involved in initiating defensive behaviours is the muscarinic acetylcholine receptor.

50-kHz ultrasonic vocalizations are correlated with positive valenced behaviours

Similarly, as with 22-kHz USVs, in order to establish that the production of 50-kHz USVs indexes positive emotional states in rats some behavioural and chemical criteria should be satisfied. Since humans report that positive pro-social contact is involved in the self-report of positive valence (Csikszentmihalyi & Hunter, 2003), the production of 50-kHz USVs should be induced by positive pro-social encounters amongst conspecifics (Knuston et al, 2002). From a pharmacological perspective, appetitive 50-kHz USVs should be induced by manipulations of

brain structures and neurotransmitters that have been reported to be associated with positive emotional valence in humans.

Behavioural changes associated with the production of 50-kHz USVs

There are primarily two pro-social circumstances in which the production of 50-kHz USVs can be initiated. These circumstances can be broken down into vocalizations that are involved in communication during sexual encounters between male and female participants and communication between conspecifics during non-sexual social encounters. During sexual encounters the 50-kHz USVs are produced by both male and female partners (Thomas & Barfield, 1985; McIntosh & Barfield, 1980; White et al, 1993) and promote sexual contact between the pair. Production of 50-kHz USVs by the male is able to increase the incidence of darting and ear-wiggling in the female (Thomas, 1981), both of which are signs of proceptivity in the female (Thomas et al, 1982). Moreover, when estrous females are placed with devocalized males (males whom had laryngeal nerve resection) their proceptive behaviour is impaired but can be restored by the playback of male 50-kHz USVs (Geyer & Barfield, 1978). In sexual situations 50-kHz USVs play a pivotal role promote receptive behaviours in the female and male rat (Wöhr & Schwarting, 2010).

Appetitive 50-kHz USVs are also produced during non-sexual pro-social contacts in rats. Strong evidence that supports such a social role for 50-kHz USVs is demonstrated by rough-and-tumble play experiments (Knutson et al., 1998). During rough-and-tumble play between adolescent rats, one rat will dart on the back of a partner with their forepaws (also known as dorsal contact) and direct a play bite to the nape of the partner's neck. The partner in defense will orient its posture into a supine position to defend against its neck from the play attacks. This allows the attacker to playfully "pin" the defender (Panksepp, et al, 1984; Siviy & Panksepp,

1987; Smith et al, 1998; Knuston et al, 2002). Rough-and-tumble play behaviour is presumed to be rewarding because animals show place preference for spaces where they have experienced play and will emit 50-kHz USVs in anticipation of play (Knuston et al, 2001). Another behavioural measure used to quantify that play is a form of appetitive behaviour is latency of approach.

Latency of approach is the duration it takes for a partner to make contact with their conspecific to induce play behaviour. The longer the time the animal takes to make dorsal contact represents less drive for the animal to engage in play behaviour. If the animal demonstrates a short latency it means the more drive the animal has to engage in play behaviour. Using "tickling" procedure, an experimenter mimics the behaviour of two adolescent rats during rough-and-tumble play by making dorsal contact with a adolescent rat and flipping of the animal into a supine position. Quantifying latency of approach of the animal during ticking and number of 50-kHz USVs Burgdorf & Panksepp (2001) found a negative correlation between numbers of 50-kHz USVs emitted and approach latency for tickling (Burgdorf & Panksepp, 2001). This means the high number of produced 50-kHz USVs, the more eager animals are to engage in contact with the experimenter.

Rats not only produce 50-kHz USVs during play but, when given the opportunity, they will eagerly search out the source of emitted 50-kHz USVs. Using an operant sound-sample self-administration chamber, Burgdorf and colleagues (2008) were able to show rats will nose-poke for continuous playback of 50-kHz USVs over tape hiss and 22-kHz USVs (Burgdorf et al., 2008). Similar results were also reported by Wöhr and Schwarting (2007). Playback of 50-kHz USVs was able to activate behavioural changes from the animal within a radial arm maze to approach the source of emitted 50-kHz USVs. This ability of 50-kHz USVs to induce approach

was not dependent on juvenile or adult male rats; however some behavioural differences did exist between the two groups. Juvenile rats approached the source of 50-kHz USVs with a smaller latency then did adult rats however, adult rats emitted 50-kHz USVs in response to playback of 50-kHz USVs. Although these behaviours were different, both behaviours still displayed the characteristics of appetitive pro-social behaviour.

In rat brain, the processing of 50-kHz USVs is associated with multiple brain structures that are not active during processing of 22-kHz USVs. Rats show increased Fos-immunoreactive protein in sites that are consistent with reward and self-stimulation such as the lateral hypothalamus and the ventral striatum (Sadananda et al, 2008). These results suggest that the signal the 50-kHz USV carries is appetitive when decoded by the receiver.

Regions of the brain associated with reward and appetitive emotional states

Electrical stimulation of the brain is a powerful experimental method to probe the contribution of various brain regions to organized behaviour. Its application and subsequent use for mapping neural structures associated with reward, and subsequently pleasure, was first demonstrated by Olds and Milner (1954) who were interested in brain regions associated with reinforcing functions. Olds and Milner demonstrated that rats would develop place-preferences behaviours when experiencing an electrical current to different brain areas. Also, animals in operant conditioning experiments would quickly learn to perform a task, such as lever pressing, to receive electrical stimulation of brain areas (Olds and Milner, 1954). This led Olds and Milner to the notion that there are distinct regions in the brain that are dedicated to reward, which are used to change and reinforce the behaviour of animals.

A partial clue to the neurochemical mediators that contribute to reward stemmed from clinical observations that schizophrenics had decreased self-reports of pleasure from rewarding stimuli (i.e. food and sex) while on anti-psychotic drugs. Animal studies employing self-administration reserpine, imipramine and chlorpromazine (Stein, 1962) attenuated rewarding self-administration behaviour in rats. This led to the hypothesis that noradrenaline was involved in the reward system. In support of this hypothesis Wise and Stein (1969) injected sodium diethyldithiocarbamate and disulfiram, drugs that inhibit dopamine-β-hydroxylase (Szmigielski, 1975), and reported that rats decreased medial forebrain bundle electrical stimulation (Wise and Stein, 1969), a behaviour associated with reinforcement and reward (Hernandez et al, 2006). This evidence was argued by Stein and colleagues as support that noradrenalin mediates reward. Anatomical and pharmacological evidence would soon refute this hypothesis and replace it with another catecholamine.

Experiments combining self-injection protocols and anatomical-mapping experiments began to contradict the noradrenalin hypothesis of reward. Yokel and Wise (1975) showed that injection of noradrenergic antagonists did not disrupt the intravenous administration of amphetamine (Yokel & Wise, 1975) or cocaine (De Wit & Wise, 1977). Also destroying the ascending dorsal noradrenergic fiber bundle was unable to disrupt intracranial self-administration in rats. Later experiments that utilized dopaminergic antagonists were able to show clear decreases in self-administration experiments and thus a decrease in reward acquisition (Rolls, 1974; Rolls et at, 1975). Slowly the noradrenalin hypothesis of hedonia was replaced with the dopamine hypothesis of motivation and hedonia (Wise et al., 1978).

The anhedonia hypothesis was a major contributor to the hypothesis regarding the function of dopamine in reward and pleasure (Wise, 2008). Dopamine containing cells are

scattered along the base of the mesencephalon and are grouped into mesocortical, mesolimbic and nigrostriatal systems (Ikemoto & Panksepp, 1999; Ikemoto, 2007). The mesocortical dopamine system contains cell bodies that are localized within the ventral tegmental area (VTA) that ascend and innervate aspects of the medial prefrontal cortex in rat. The nigrostriatal system contains dopamine cell bodies that are localized in the substantia nigra and ascend to innervate aspects of the striatum, a major input structure for the basal ganglia (Feldman et al, 1997). This pathway plays an important regulation in locomotor behaviour since damage to this system can cause Parkinsonism-like behaviours (Ungerstedt & Arbuthnott, 1970). Finally the mesolimbic system contains dopamine cell fibers that originate within the ventral tegmental area and ascend through the medial forebrain bundle to terminate in forebrain regions responsible for motivation and emotional regulation in the animal (Feldman et al, 1997). These brain sites include the ventral striatum, more specifically nucleus accumbens, amygdala, and hippocampus. The mesolimbic dopamine system has been intensively investigated because its pathophysiology is implicated in addiction.

The striatum and its ventral division

The striatum can be divided into a ventral and dorsal tier. The dorsal striatum comprises the caudate-putamen and receives the majority of afferents from glutamatergic pyramidal cells of the cortical mantle (Delong & Wichmann, 2007). The excitatory input from the cortical mantle converges onto interneurons and medium spiny-projection neurons that in turn influence the activity of motor related nuclei such as the dorsal pallidum, and subthalamic nucleus (Afifl, 1994). The dorsal striatum has thus been categorized as belonging to the basal ganglia, a set of nuclei involved motor sequencing (Gerfen and Wilson, 1996).

The ventral extension of the caudate-putamen is known as the ventral striatum and includes the nucleus accumbens and olfactory tubercle (Zahm & Brog, 1992). Although the olfactory tubercle is involved in the classification of the ventral striatum, this nucleus will not be included when talking about the divisions of the nucleus accumbens in this thesis, and subsequent usage of the ventral striatum will only refer to the nucleus accumbens. The nucleus accumbens is a sheet of gray matter located in the rat forebrain. In its caudal aspect it merges without clear distinction with the extended amygdala, is bounded by the external capsule in rostral and lateral directions (Ikemoto, 2007) and medially by the lateral ventricles and the septum (Humphries and Prescott, 2010). Based on anatomical and histochemical markers the nucleus accumbens can be divided into core and shell regions.

Histochemical markers can distinguish between core and shell regions of the nucleus accumbens (Zaborszky et al, 1985). The core of the nucleus accumbens stains densely for the calbindin, while the shell of the accumbens stains heavily for the calcium binding protein calretinin, and acetylcholine esterase (Bubser et al, 2000). These differences in histochemical organization were correctly argued to represent different functional units. Analyzing the afferent-efferent relationship of the core of the nucleus accumbens revealed connections with brain nuclei involved motor processing such as the subthalamic nucleus, while the shell of the nucleus accumbens revealed connections with limbic structures involved in motivation and emotional regulation, such as the VTA (French and Totterdell, 2003; Goto & Grace, 2005; Heimer et al., 1991).

Relationship between the VTA and the shell of the nucleus accumbens during self-administration tests

Electrical stimulation of the MFB or the lateral hypothalamus in rats has shown to produce behaviours indicative of positive valence and reward (Olds and Milner, 1954). Support that dopamine release in the nucleus accumbens is involved in rewarding aspects of electrical stimulation comes from experiments employing chronoamperometry and high performance liquid chromatography. Chronoamperometry is able to measure dopamine oxidation currents while high performance liquid chromatography is able to measure the dopamine metabolites 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA). Both forms of neurochemical detection showed increases in dopamine release in the shell of the nucleus accumbens in response to rewarding self-administration of electrical current to the MFB and VTA (Fibiger et al, 1987; Blaha & Philips, 1990; Philips et al., 1992). Interestingly, drugs that are abused by humans for their hedonic value (opiates, ethanol, nicotine, amphetamine, and cocaine; Bock et al, 2007), are all able to increase the endogenous levels of dopamine within the nucleus accumbens of the rat when injected systemically (Di Chiara & Imperato, 1988) and a subset of these compounds that are abused by humans can produce 50-kHz USVs in rats (Thompson et al, 2006).

Damage to the nucleus accumbens can disrupt self-administration of drugs in rats. Injection of 6-hydroxydopamine, a specific dopamine neurotoxin, bilaterally into the nucleus accumbens disrupts the acquisition and maintenance of self-administration of *d*-amphetamine without deficits in locomotor behaviour (Lyness et al, 1979). Destruction of dopamine fibers also diminishes the rates of cocaine self-administration (Roberts & Koob, 1980) and decreases the

rate of MFB self-stimulation (Steller & Corbett, 1989) suggesting the integrity of the mesolimbic dopamine system is needed for reward aquisition.

The chemical constituents responsible for the production of 50-kHz USVs in rats

If the dopamine hypothesis of pleasure and positive affect is correct, then ingestion of drugs of abuse that are commonly associated with euphoria in human subjects should increase dopamine transmission within the mesolimbic dopamine system of the rat. Drevets and colleagues (2001) combined positron emission tomography to correlate changes in dopamine levels following amphetamine administration with hedonic self-reports. The authors reported that amphetamine increases endogenous dopamine levels within the ventral striatum of human subjects in response to amphetamine ingestion, and this increase in dopamine release is positively correlated to hedonic responses in the subjects (Drevets et al., 2001). Since amphetamine increases the self-indexed report of hedonia in human subjects, then injection of amphetamine into the ventral striatum of the rat should produce robust, species-typical 50-kHz USVs since this vocalization indexes appetitive states in the rat. Results reported by Thompson and colleagues (2006) and Burgdorf and colleagues (2001) support such a prediction. Thompson and colleagues (2006) reported the production of 50-kHz USVs in response to amphetamine injections into the shell of the nucleus accumbens during a quantitative mapping study. This increase in 50-kHz USVs was mediated by dopamine since pretreatment of the nucleus accumbens with SKF-83566 or raclopride, both dopamine antagonists, reduced the number of recorded 50-kHz USVs (Thompson et al, 2006). The results of Thompson and colleagues are consistent with Burgdorf and colleagues, who also reported that amphetamine increases the production of 50-kHz USVs when injected into the medial shell of the nucleus accumbens (Burgdorf, 2001).

Further support that dopamine regulates the initiation of appetitive 50-kHz USVs comes from recording of 50-kHz USVs during anticipation of rewarding situations. Burgdorf and colleagues (2000) showed that rats, when anticipating an electrical stimulation of the VTA or the lateral hypothalamus (LH), showed significant increases in the production of 50-kHz USVs (Burgdorf et al., 2000). Injection of raclopride, a dopamine receptor antagonist, decreases the number of 50-kHz USVs (Thompson et al, 2006) in response to both rough-and-tumble play and in anticipation of rewarding brain stimulation (Burgdorf et al., 2007). Systemic injections of flupenthixol, another type of dopamine receptor antagonist, produces deficits in the production of 50-kHz USVs in response to "tickling" and anticipation of electrical stimulation in a similar manner to animals that have had electrolytic lesions placed in the VTA (Burgdorf et al, 2007).

Closing remarks on dopamine and the production of 50-kHz USVs

The production of 50-kHz USVs indexes an appetitive state within the signaler. These vocalizations are produced under positive pro-social circumstances and can be pharmacologically induced by the systemic injections of drugs of abuse, or when injected into the ventral striatum. The messages that these vocalizations carry seem to be involved in the maintenance or re-establishment of social contact amongst conspecifics since they are used during mating and adolescent play and in response to playback recordings of 50-kHz USVs. These vocalizations are also accompanied by approach like locomotor changes in the receiver of the animal towards the source of emitted 50-kHz. These behaviours are opposite reactions to animals responding to 22-kHz USVs. Thus, 50-kHz USVs are produced under appetitive circumstances and can index the appetitive emotional state in the signaler.

The ascending mesolimbic dopamine system and the ascending mesolimbic cholinergic system

The production of 50-kHz USVs and the production of 22-kHz USVs are dependent upon two separate neurochemical systems. The production of appetitive 50-kHz USVs is dependent upon the release of dopamine into the medial shell of the nucleus accumbens, while the production of 22-kHz USVs is dependent upon acetylcholine being released into the terminal fields of the medial cholinoceptive vocalization strip. These two ascending systems in the rat represent two functionally separate emotional systems. Functionally, this means that during the production of 50-kHz USVs the probability of producing 22-kHz USVs is diminished and vice versa.

Electrophysiological evidence also indirectly suggests these two systems function in opposition to each other. Since the ascending mesolimbic dopamine system is involved in behavioural responses associated with appetitive emotions, these dopamine containing cell bodies within the VTA should be inhibited during aversive situation. Indeed, there have been many electrophysiological reports showing such a relationship. Mileykovskiy and Morales (2011) recorded the electrophysiological behaviour of tyrosine hydroxylase positive neurons (TH+; a marker for dopamine neurons) in the parabrachial pigmented and paranigral nuclei (part of the ventral tegmental area) to aversive fear conditioning. The authors reported significant decreases in the discharge rates of dopamine neurons during anticipation of an electrical tail shock and decreased respiratory activity of the animals (Mileykovskiy and Morales, 2011). These results are similar with Mirenowicz and Schultz study reporting a preferential activation of dopamine containing VTA neurons to rewarding stimulation and a preferential inhibition of dopamine containing VTA neurons to negative stimulation (Mirenowicz and Schultz, 1996).

These results suggest an inverse relationship between reward and punishment responses encoded by the properties of dopamine neurons within the VTA.

Despite the work involving the different circumstances in which the production of 22-kHz or 50-kHz USVs can be emitted, no investigation has sought out to establish a relationship between the two neural systems that are responsible for the production of either 22-kHz USVs or 50-kHz USVs. So far the work has only been indirect. This thesis focused on investigating the relationship between the two neural systems responsible for both emotional expression states in rats.

Central Question

If the mesolimbic dopamine system and the mesolimbic cholinergic system represent two different pathways regulating different emotional states in rats, then do these systems interact? And if so, what is the nature of this interaction? Another question that was investigated throughout this thesis was if the parameters of 22-kHz or 50-kHz USVs changed upon activation of the antagonistic systems.

Hypothesis

The production of 22-kHz USVs indexes emotionally aversive states in the signaler. The production of aversive 22-kHz is dependent upon the release of acetylcholine into the terminal fields along the medial cholinoceptive vocalization strip. The production of 50-kHz USVs indexes an emotionally appetitive state in the signaler and can be initiated by injection of dopamine agonists into the medial shell of the nucleus accumbens. Since these two systems are functionally antagonistic to each other, activation of the ascending mesolimbic dopamine system, before injection of carbachol into the medial cholinoceptive vocalization strip, will attenuate the

number of 22-kHz USVs reflecting a functional antagonism of the aversive emotional state. Conversely, activation of the ascending mesolimbic cholinergic system involved in negative emotional states will attenuate the number of 50-kHz USVs induced by injection of a dopamine agonist into the medial shell of the nucleus accumbens, reflecting a functional antagonism of an appetitive emotional state.

Although the two systems governing the emotional expression of 22-kHz USVs and 50-kHz USVs are antagonistic to each other, the characterization of emitted USVs will not change in any condition because the duration and peak frequency of the USVs is critical for properly transmitting biologically important information.

Goals and Predictions

The goal of this thesis was to provide evidence of the mutually antagonist relationship between the aversive-emotional system responsible for the production of 22-kHz USVs and the appetitive-emotional system responsible for the production of 50-kHz USVs. Activation of the ascending mesolimbic dopamine system was done by injection of apomorphine into the medial shell of the nucleus accumbens while activation of the ascending mesolimbic cholinergic system was done by injection of carbachol into the AH-MPO and the LS. The following predictions were proposed based on the functional antagonistic relationship of the ascending mesolimbic dopamine system and ascending mesolimbic cholinergic system:

1) Apomorphine, when injected into the shell of the nucleus accumbens, will dose-dependently induce the emission of 50-kHz USVs
2) Carbachol-induced 22-kHz USVs from the AH-MPO or LS will be blocked or decreased by *earlier* injection of apomorphine into the medial shell of the nucleus accumbens

3) Apomorphine induced 50-kHz USVs from the medial shell of the nucleus accumbens will be blocked or decreased by *earlier* injection of carbachol into the AH-MPO or the LS
4) Antagonism of the emotional systems will not change the parameters that define the characteristics of aversive 22-kHz USVs or appetitive 50-kHz USVs.

Rationale for apomorphine use

Amphetamine is a potent pharmacological compound that can increase the endogenous level of dopamine with the nucleus accumbens and can unconditionally initiate the production of 50-kHz USVs (Burgdorf et al, 2001). However, the range of pharmacological compounds classified as dopamine agonists which can initiate 50-kHz USVs have been limited to amphetamine or cocaine (which was given systemically). In this thesis another dopamine agonist, apomorphine, will be used to see if it can also initiate 50-kHz USVs in rats. This will provide further support to the hypothesis that dopamine release in the nucleus accumbens is sufficient for the initiation of 50-kHz USVs.

Enantiomers of apomorphine, R-(-)-apomorphine and S-(-)-apomorphine, are widely studied class of pharmacological alkaloids with broad spectrum affinity for both D_1 and D_2 class of dopamine receptors (Kula et al, 1985; Saller & Salama, 1986). In binding assays, it has been demonstrated that the R-(-) configuration of apomorphine can stimulate dopamine receptor-mediated adenyl-cyclase (Kula et al., 1985), activate dopamine autoreceptors located on the soma of dopamine neurons (Saller & Salama, 1986) and can compete with spiroperidol, a known dopamine antagonist (Kula et al., 1985). Actions of the R-(-) configuration can be antagonized by co-application of dopamine receptor antagonists like haloperidol and sulpride, suggesting that R-(-) configuration functions by binding to dopamine receptors and initiating biological cascades that are akin to dopamine binding to dopamine receptors. Behavioural experiments testing the

locomotor activity of animals injected with apomorphine show similar behvioural patterns to animals injected with amphetamine. Animals, when injected with the R-(-) configuration show increased cage climbing (Wilcox et al, 1979), increased locomotor activity (Kiyatkin, 1994) and increased gnawing compulsion in rodents (Ernst, 1967). These behavioural and biochemical responses were not observed with the S-(-)-apomorphine (Campell et al., 1984). Since the behaviours produced by R-(-)-apomorphine are similar to those produced by amphetamine, and since amphetamine can initiate the production of 50-kHz USVs when injected systemically or into the shell of the nucleus accumbens, this thesis will focus on R-(-)-apomorphine and its ability to produce 50-kHz USVs.

To initiate the production of aversive 22-kHz USVs in rats for this thesis, 1.0 µg of carbachol (a muscarinic cholinergic agonist) will be injected into the terminal fields of the medial cholinoceptive vocalization strip localized to the AH-MPO and the LS. Dose-response curves indicate that 1.0 µg is able to induce the maximal response of emitted 22-kHz USVs (Bihari et al, 2003).

Methods and Materials

Experimental Subjects

The animals used throughout this thesis were 60 adult, male Long Evans rats weighing between 250-370 grams. Upon arrival to the Brock University animal facility from Charles River, Montreal Quebec, the animals were housed in 560 mm x 250 mm x 195 mm polycarbonate cages for three days to acclimate to the new facility. The animals were housed with one large black polyvinyl tube and 4 sheets of paper towel to provide hiding place and to enrich their environment. All experimental procedures carried out on the animal subjects were

done in accordance with the Canadian Council on Animal Care (CCAC) and were approved by the Brock Animal Care Committee. All surgical procedures and post-operative care were done with the supervision of an appointed veterinarian. Experimentations began five days after surgical procedure on healthy post-operative rats. All rats were healthy throughout the study and showed species-typical behaviour.

Stereotaxic Surgeries

For intracerebral injections, rats needed to have cannulae surgically implanted into their brain. Rats were prepared for surgery by receiving an intraperitoneal mixture of ketamine (40 mg/kg, MTC Pharmaceuticals, Cambridge, Ontario) and xylazine hydrochloride (6 mg/kg, Chemargo Ltd., Ontario). After proper sedation and depth of anesthesia, the animals received an injection of trimethoprim and sulfadiazine (1:5) at a dose of 240 mg/kg to prevent bacterial infections and metacam (2 mg/kg) to provide pain management. Under full anesthesia, the animal's heads were shaved and then placed into a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Once properly mounted, the surgical site was scrubbed with 7% iodine solution combined with detergent. The detergent was cleared off the scalp by 70% isopropanol and then the area was locally re-sterilized with 10% iodine. After proper skin treatment, an incision was made with a # 20 scalpel blade in the rostro-caudal direction. After the incision was made, the periosteum was scraped away revealing the underlying skull and its anatomical features. The arm of the stereotaxic device was then calibrated according to the position of lambda stereotaxic zero on the surface of the skull. After small holes were drilled into the skull, 23 gauge stainless-steel guide cannula were unilaterally implanted into the left nucleus accumbens shell (A=9.7 – 10.33 mm, L= 0.8-1.3 mm, V= - 6.4 to -6.6 mm), left anterior hypothalamic-medial preoptic area (A = 7.8 to 8.4 mm, L=0.2 to 0.8 mm, V= -8.5 to -8.8 mm) or

left lateral septum (A=9.6 to 9.8 mm, L=0.4 to 0.6 mm, V= -4.5 to -5.2 mm). All coordinates were taken from lambda (Paxinos & Watson, 1986).

Following cannulae implantation, methyl methylacrylate resin (Hygenic Corporation of Canada, St. Catharines, Ontario) was used to permanently secure the implant to the skull. The opening of the cannula was blocked by inserting a stainless steel wire plug into the guide cannula. After surgery, the animal was given the analgesic meloxicam at a dose of 2-5 mg/kg s.c. Animals were then placed in post-surgery cages suspended on a grate. After the animal had recovered, they were placed back into their pre-surgical environments but housed individually.

Animals were exposed to handling and left to recover over a five day period. Surgeries were completed within 3 days for each experimental group.

Drugs and Intracerebral Injections

R-(-)-apomorphine hydrochloride hemihydrate was purchased from Sigma-Aldrich Quebec Canada. R-(-)-apomorphine hydrochloride hemihydrate was prepared in a solution containing 1% ascorbic acid dissolved in pyrogen-free sterile saline to 3.0 µg/0.3 µl. The contents were kept away from light, heat, and air by wrapping it in tin-foil and storing in the refrigerator. Carbamylcholine chloride was purchased from Sigma-Aldrich Quebec Canada. Carbamycholine chloride was dissolved in a pyrogen-free sterile saline to a dose of to 1.0 µg/0.3 µl. For control injections a 1% ascorbic acid solution was prepared in a pyrogen-free sterile saline.

All intracerebral injections were organized in a partly counter balanced manner. The first 15 rats were used to obtain the dose-response relationship between number of 50-kHz USVs and dose of apomorphine injected into the shell of the nucleus accumbens (Table 1). The subsequent injection groups were divided into five injection schedules as outlined in Tables 2-5. All injections were completed using a Hamilton constant rate syringe (CR-700) and polyethylene tubing connected to a 30 gauge stinless steel injection cannula. The total volume of injection was 0.3 µl per site and a time delay of one minute was given in between double injections before recording USVs. The double injections were conducted every 3 days.

Table 1:

Dose Response injection schedule for apomorphine

Rat I.D	Injection 1	Rat I.D	Injection 2	Rat ID	Injection 3	Rat ID	Injection 4	Rat ID	Injection 5
RMM1	3.0 µg	RMM15	3.0 µg	RMM11	3.0 µg	RMM6	1.0 µg	RMM7	2.5 µg
RMM2	1.0 µg	RMM14	0.5 µg	RMM10	2.5 µg	RMM5	2.5 µg	RMM3	1.0 µg
RMM3	2.5 µg	RMM13	2.5 µg	RMM9	1.75 µg	RMM10	1.0 µg	RMM9	0.5 µg
RMM4	1.75 µg	RMM12	3.0 µg	RMM12	1.75 µg	RMM11	1.0 µg	RMM14	1.0 µg
RMM5	3.0 µg	RMM11	1.75 µg	RMM13	1.75 µg	RMM4	2.5 µg	RMM1	2.5 µg
RMM6	1.75 µg	RMM10	0.5 µg	RMM14	3.0 µg	RMM3	3.0µg	RMM6	2.5 µg
RMM7	1.0 µg	RMM9	3.0 µg	RMM15	2.5 µg	RMM12	1.0 µg	RMM2	0.5 µg
RMM8	0.5 µg	RMM8	1.75 µg	RMM1	1.0 µg	RMM13	1.0 µg	RMM11	2.5 µg
RMM9	2.5 µg	RMM7	0.5 µg	RMM2	1.75 µg	RMM7	3.0 µg	RMM10	1.75 µg
RMM10	3.0 µg	RMM6	3.0 µg	RMM3	1.75 µg	RMM8	1.0 µg	RMM4	0.5 µg
RMM11	0.5 µg	RMM5	1.75 µg	RMM4	1.0 µg	RMM14	1.7.5 µg	RMM13	3.0 µg
RMM12	2.5 µg	RMM4	3.0 µg	RMM5	1.0 µg	RMM15	0.5 µg	RMM12	0.5 µg
RMM13	0.5 µg	RMM3	0.5 µg	RMM6	0.5 µg	RMM9	1.0 µg	RMM15	1.75 µg
RMM14	2.5 µg	RMM2	2.5 µg	RMM7	1.75 µg	RMM1	1.75 µg	RMM5	0.5 µg
RMM15	1.0 µg	RMM1	0.5 µg	RMM8	2.5 µg	RMM2	3.0µg	RMM8	3.0 µg

Table 2:

Injection schedule for injection set 1a

Animal I.D #	sal+carb	sal+sal	apo+carb	apo+sal
Injection 1	Rat 16, 19, 22	Rat 18, 25	Rat 21, 26, 20	Rat 23,17, 24
Injection 2	Rat 17, 23, 24	Rat 22, 16, 19	Rat 25, 18	Rat 26, 21, 20
Injection 3	Rat 20, 21, 26	Rat 24, 17, 23	Rat 19, 22, 16	Rat 18, 25
Injection 4	Rat 25, 18	Rat 26, 20, 21	Rat 23, 24, 17	Rat 16, 22, 19

Table 3:

Injection schedule for injection set 1b

Animal I.D #	sal+carb	sal+sal	apo+carb	apo+sal
Injection 1	Rat 27, 30, 33	Rat 29, 38, 36	Rat 32, 37, 31	Rat 35, 34, 28
Injection 2	Rat 28, 34, 35	Rat 33, 27, 30	Rat 36, 29, 38	Rat 37, 32, 31
Injection 3	Rat 31, 32, 37	Rat 35, 28, 34	Rat 30, 33, 27	Rat 29, 36, 38
Injection 4	Rat 38, 36, 29	Rat 37, 31, 32	Rat 34, 35, 28	Rat 33, 30, 27

Table 4:

Injection schedule for experiment 2a

Animal I.D #	carb+sal	sal+sal	carb+apo	sal+apo
Injection 1	Rat 39, 42, 45	Rat 50, 41, 48	Rat 44, 49, 43	Rat 47, 46, 40
Injection 2	Rat 40, 46, 47	Rat 45 39, 42	Rat 48, 50, 41	Rat 49, 44, 43
Injection 3	Rat 43, 44, 49	Rat 47, 40, 46	Rat 42, 45, 39	Rat 50, 48, 41
Injection 4	Rat 41, 48, 50	Rat 49, 43, 44	Rat 46, 47, 40	Rat 45, 39, 42

Table 5:

Injection schedule for experiment 2b

Animal I.D #	carb+sal	sal+sal	carb+apo	sal+apo
Injection 1	Rat 51, 54, 57	Rat 53, 60	Rat 55, 56	Rat 59, 58, 52
Injection 2	Rat 52, 58, 59	Rat 57, 51, 54	Rat 60, 53	Rat 56, 55
Injection 3	Rat 55, 56	Rat 59, 52, 58	Rat 55, 57, 51	Rat 53, 60
Injection 4	Rat 60, 53	Rat 56, 55	Rat 58, 59, 52	Rat 57, 54, 51

The four different injection groups

After the suitable dose of apomorphine was established to elicit 50-kHz USVs, the rats were separated into four different injection set: injection set 1a, 1b and injection set 2a and 2b. The first set of injections were to investigate if *prior* injection of apomorphine into the shell of the nucleus accumbens could decrease the number of carbachol induced 22-kHz USVs from the AH-MPO (set 1a) or the LS (set 1b). The second sets of injections were to investigate if *prior* injection of carbachol into the AH-MPO or the LS could decrease the number of apomorphine induced 50-kHz USVs when injected into the shell of the nucleus accumbens. In all injection groups the call parameters across all conditions were quantified in order to confirm the fourth prediction.

Injection set 1a

This experiment focused on antagonizing carbachol-induced 22-kHz USVs elicited from the AH-MPO by *prior* injection of apomorphine into the medial shell of the nucleus accumbens. Order of injections are illustrated in Figure 1. Injection conditions for this group were as follows: 1) Saline injection into the medial shell of the nucleus accumbens occurred one minute prior to injection of carbachol into the AH-MPO (sal+carb). 2) Apomorphine was injected into the medial shell of the nucleus accumbens one minute before saline was injected into the AH-MPO (apo+sal). 3) Apomorphine was injected into the medial shell of the nucleus accumbens one minute before injection of carbachol into the AH-MPO (apo+carb). 4) Saline was injected into the shell of the nucleus accumbens one minute prior to injection of saline into the AH-MPO (sal+sal). For sequence of injections for each animal see Table 2.

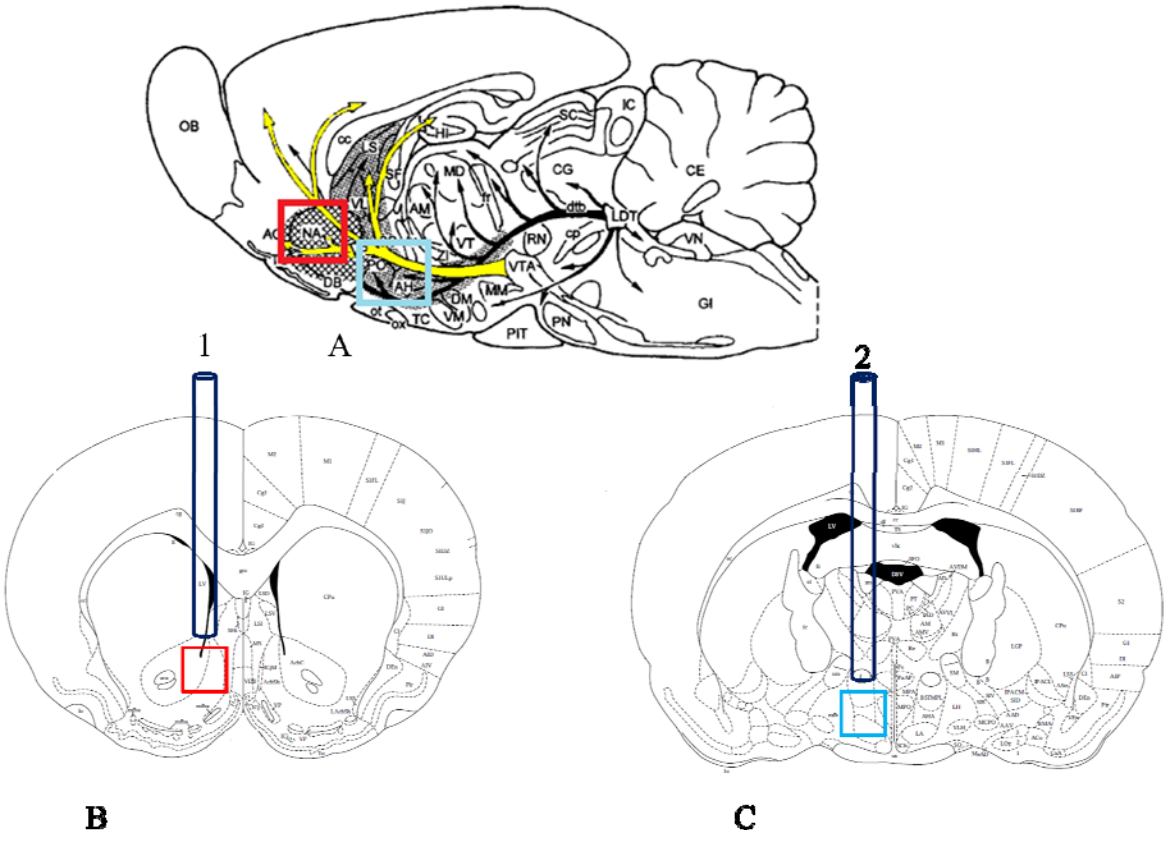

Figure 1: Overview of injection set 1a. (A) The parasaggital cross section of the rat brain showing both the ascending mesolimbic cholinergic system innervating the medial cholinoceptive vocalization strip responsible for the initiation of 22-kHz USVs (black; shaded) and the ascending mesolimbic dopamine system responsible for the initiation of 50-kHz USVs (yellow; cross-hatched). (B). Coronal section of the rat brain showing the location of apomorphine injection localized into the nucleus accumbens shell (red square). (C). Coronal section of the rat brain showing the location of carbachol injection localized to the AH-MPO and the location of the second injection (blue square). The injection into the sNa will activate the target areas of the ascending mesolimbic dopamine system and the injection into the AH-MPO will activate the target areas of the ascending mesolimbic cholinergic system.

Injection set 1b

This series of experiments focused on antagonizing carbachol-induced 22-kHz USVs from the lateral septum (LS) by *prior* injection of apomorphine into the medial shell of the nucleus accumbens (Figure 2). The order of injections for this experimental group was as follows: 1) Saline was injected into the medial shell of the nucleus accumbens prior to injection of carbachol into the LS (sal+carb). 2) Apomorphine was injected into the medial shell of the nucleus accumbens prior to injection of saline into the LS (apo+sal). 3). Apomorphine was injected into the medial shell of the nucleus accumbens one minute prior to injection of carbachol into the LS (apo+carb). 4). Saline injection into the medial shell of the nucleus accumbens occurred one minute prior to saline injection into the LS (sal+sal). For injection sequence of each animal see Table 3.

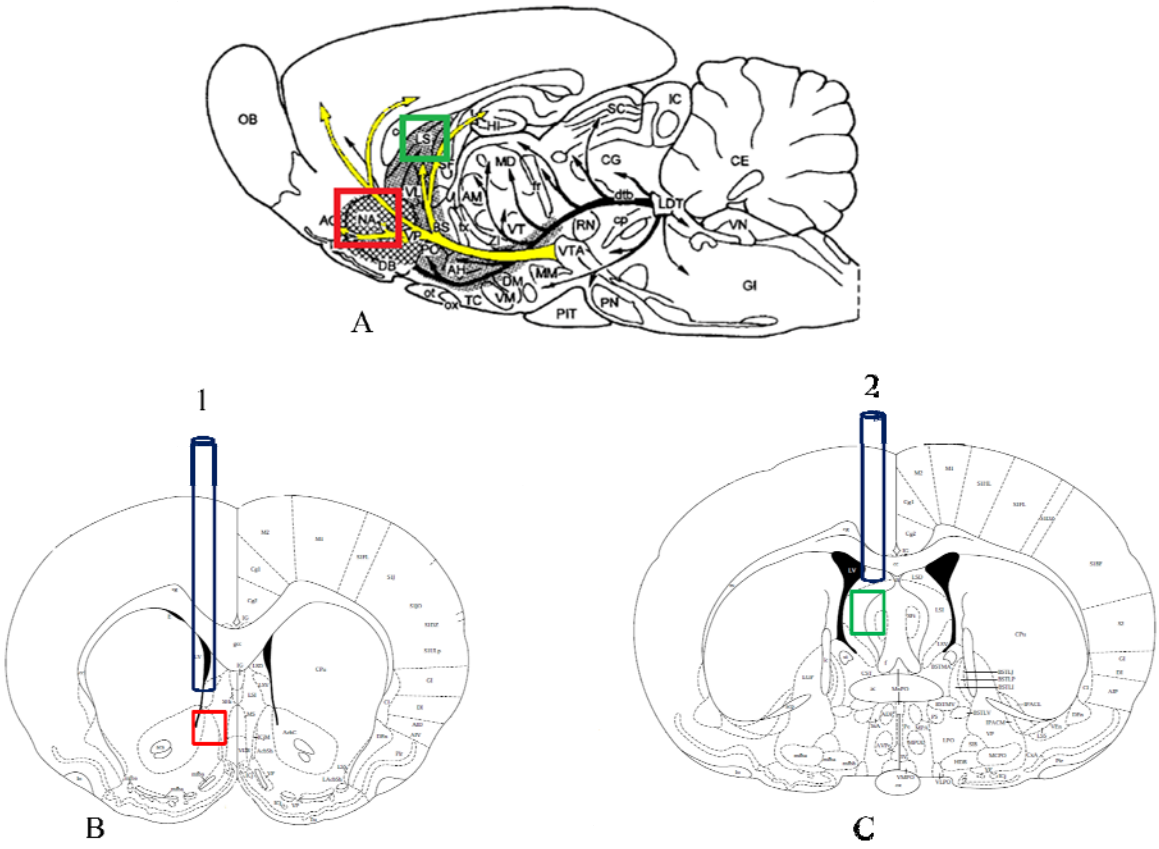

Figure 2: Overview of injections for experiment set 1b. (A) The parasaggital cross section of the rat brain showing both the ascending mesolimbic cholinergic system innervating the medial cholinoceptive vocalization strip responsible for the initiation of 22-kHz USVs (black; shaded) and the ascending mesolimbic dopamine system responsible for the initiation of 50-kHz USVs (yellow; cross-hatched). (B). Coronal section of the rat brain showing the location of apomorphine injection localized into the nucleus accumbens shell (red square). (C). Coronal section of the rat brain showing the location of the LS and subsequently the location of carbachol to be injected (green square). The injection into the sNa will activate the target areas of the ascending mesolimbic dopamine system and the injection into the LS will activate the target areas of the ascending mesolimbic cholinergic system

.

Injection set 2a

This series of experiments focused on antagonizing apomorphine induced 50-kHz USVs by *prior* injection of carbachol into the AH-MPO (Figure 3). Order of injections for this experiment was as follows: 1) Carbachol was injected into the AH-MPO one minute prior to injection of saline into the medial shell of the nucleus accumbens (carb+sal). 2) Saline injected into the AH-MPO occurred one minute prior to injection of apomorphine into the medial shell of the nucleus accumbens (sal+apo). 3) Injection of carbachol into the AH-MPO occurred one minute prior to injection of apomorphine into the shell of the nucleus accumbens (carb+apo). 4) Saline injected into the AH-MPO occurred one minute prior to injection of saline into the medial shell of the nucleus accumbens (sal+sal). For overview of animal injection conditions see Table 4.

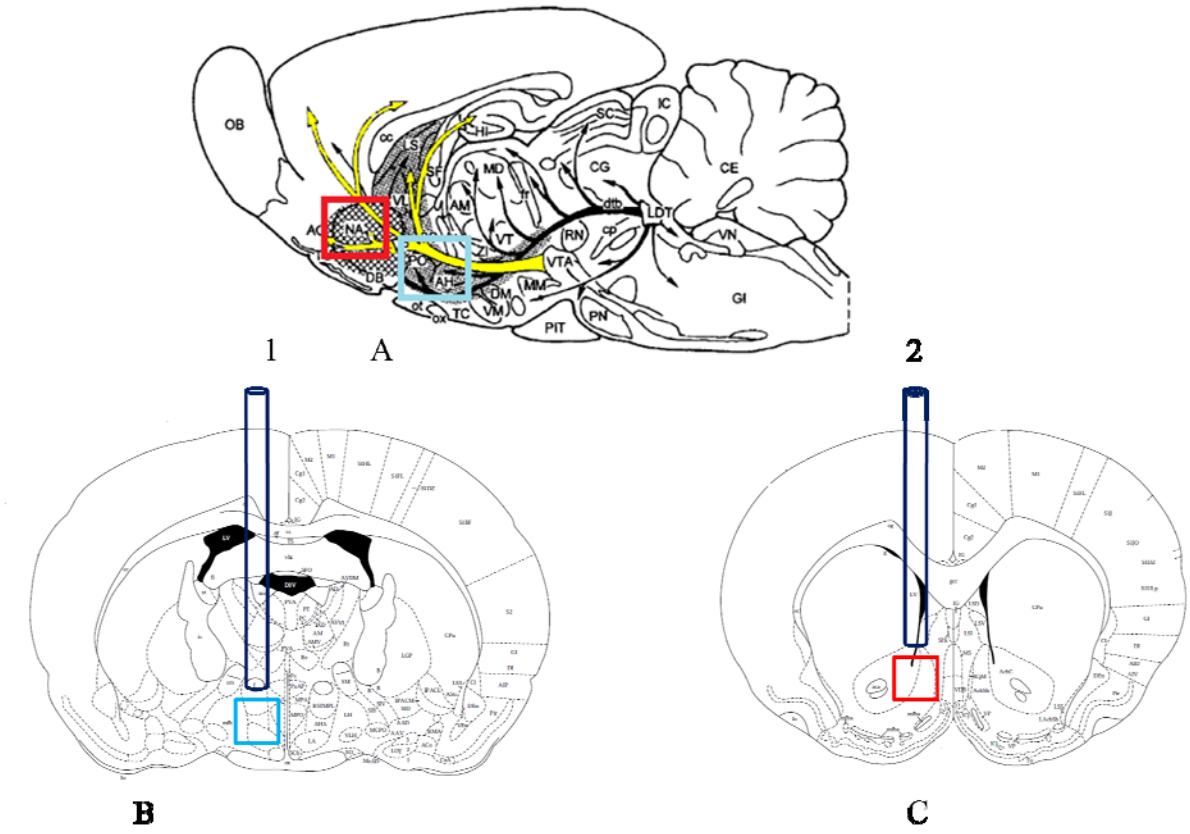

Figure 3: Overview of injections set 2a. (A) The parasaggital cross section of a rat brain showing both the ascending mesolimbic cholinergic system innervating the medial cholinoceptive vocalization strip responsible for the initiation of 22-kHz USVs (black; shaded) and ascending mesolimbic dopamine system responsible for the initiation of 50-kHz USVs (yellow; cross-hatched). (B). Coronal section of the rat brain showing the location for the first injection of carbachol into the AH-MPO (blue square) (C). Coronal section of the rat brain showing the location of apomorphine injection into the medial shell of the nucleus accumbens (red square). The injection into the AH-MPO will activate the target areas of the ascending mesolimbic cholinergic system and the injection into the medial shell of the nucleus accumbens will activate the target areas of the ascending mesolimbic dopaminergic system.

Injection set 2b

This series of experiments focused on antagonizing apomorphine induced 50-kHz USVs by *prior* injection of carbachol into the LS (Figure 4). Order of injections for this experiment was as follows: 1) Carbachol was injected into the LS one minute prior to injection of saline into the medial shell of the nucleus accumbens (carb+sal). 2) Saline injected into the LS occurred one minute prior to injection of apomorphine into the medial shell of the nucleus accumbens (sal+apo). 3) Carbachol injection into the LS occurred one minute prior to injection of apomorphine into the medial shell of the nucleus accumbens (carb+apo). 4). Saline was injected into the LS one minute before saline was injected into the medial shell of the nucleus accumbens (sal+sal). Animal injection schedules see Table 5.

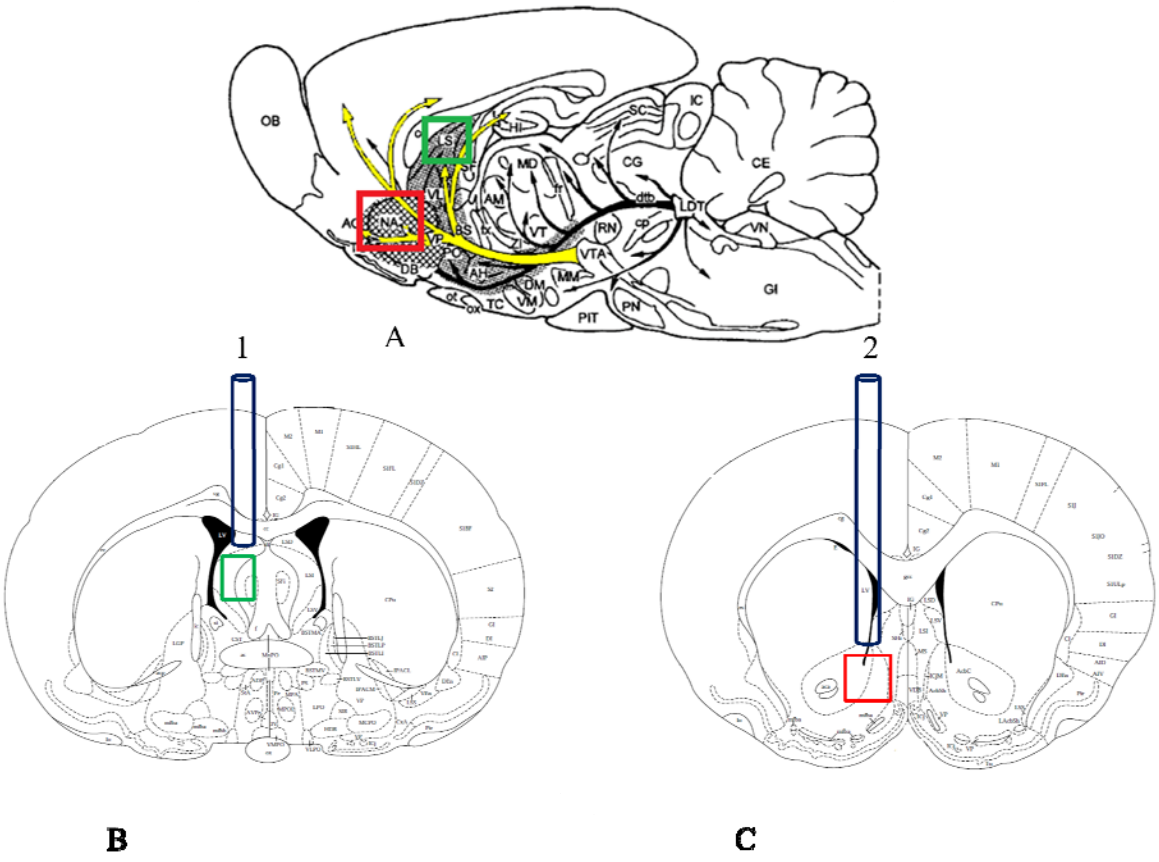

Figure 4: Overview of injections for experiment set 2b. (A) The parasaggital cross section of the rat brain showing both the ascending mesolimbic cholinergic system innervating the medial cholinoceptive vocalization strip responsible for the initiation of 22-kHz USVs (black; shaded) and the ascending mesolimbic dopamine system responsible for the initiation of 50-kHz USVs (yellow; cross-hatched). (B). Coronal section of the rat brain showing the location carbachol injection into the LS (green rectangle). (C). Coronal section of the rat brain showing the location of the second injection localized to the medial shell of the nucleus accumbens (red square). The injection of carbachol into the LS will activate the target areas of the ascending mesolimbic cholinergic system and the injection apomorphine into the sNa will activate the target areas of the ascending mesolimbic dopaminergic system.

Ultrasonic vocalization recording

Vocalization recording for all groups took place 1 minute after the injection cannula was taken out of the brain. All recordings took place in a clean polycarbonate cage (220 mm x 200 x 190 mm) lined with a single paper towel. The recording chamber was covered with wire lid supporting a condenser microphone model CM16/CPA from Avisoft Bioacoustics (Berlin, Germany) positioned about 200 mm from the animal. A dishwasher clean cage was given for each animal to elimate cross contamination with odors. All recordings were 10 minutes in duration with a sampling rate of 200, 000 Hz stored in a 16 bit format using Avisoft Bioacoustics system (Avisoft Recorder NI-DAQMX) and subsequently stored on the computer hard drive in the analysis room.

Acoustic Analysis

All acoustic files were analyzed off-line using Avisoft SASlab Pro program (Avisoft) for both 50-kHz and 22-kHz USVs recorded within the first five minutes of the testing session. A fast Fourier transformation with a temporal resolution of 488 Hz was used to deconstruct the entire 10 minute recording session into its harmonic components allowing the recorded spectral parameters of the USVs to be easily visualized and subsequently analyzed. Analysis of vocalizations focused on peak frequency (kHz) and duration (ms) of single calls. Calls and their subsequent classification were based on a study by Brudzynski (2007).

Histological procedures

After the cycles of injections were finished for each group, animals were sacrificed using an over-dose of sodium pentobarbital (240 mg/ml) at 120-150 mg/rat. When the animal was deeply anesthetized, the animal was transcardially perfused with 10% formalin solution to fix the

brain before extraction. After the brain was extracted and submerged in formalin solution for 48 hours, they were blocked and mounted on the table of the freezing microtome (Cryo-Histomat) with Tissue Tek compound. Brains were sectioned for an approximate 40 μm preparation. After sectioning, brains were placed on a polylysine coated microscope slide. The preparations were then allowed to dry for approximately 24 hours.

After the brain preparations were dried, the sections were stained using the Rucker-Koithan method, a modified version of the Nissl staining technique (Windle et al, 1943; Skinner, 1971). Nissl bodies, nuclei and necrotic tissue outlining the cannula and the injection site were stained dark blue and non-necrotic tissue in a transparent blue. After staining was complete, slides were cover-slipped using Permount glue (Fisher Scientific, Suwanee, GA, USA). Once dry, localization of injection sites were mapped using a projection light microscope on matching coronal sections of the rat brain from the atlas by Paxinos and Watson (1986).

Statistical Analysis

Analyzes of the number of ultrasonic vocalizations were performed with non-parametric Friedman's ANOVA with paired Wilcoxon Signed Ranks post-hoc test. To analyze the duration and the peak-frequency of recorded 22-kHz vocalizations, four animals were chosen: one rat from injection set 1a, one rat from injection set 1b, one rat from injection set 2a, and one rat from injection set 2b. To analyze 22-kHz USVs parameters the first 14 vocalizations (from each injection condition) from each rat were chosen then subsequently analyzed. To analyze the parameters of 50-kHz USVs (peak-frequency and duration) four animals were chosen, one from each of the injection sets. The four animals had the first five 50-kHz USVs analyzed across each injection condition while four animals had the first 14 22-kHz USVs analyzed across each

injection condition. That means for each injection set, fifty-six (14 USVs x 4 injection conditions) 22-kHz USVs were analyzed and twenty (5 USVs x 4 injection conditions) 50-kHz USVs were analyzed. All statistical calculations were non-parametric and performed with SPSS v 17.0.

Results

Dose-response study of apomorphine

Localization of injection sites for apomorphine are displayed in Figure 5. Intraaccumbens injection of five different doses of apomorphine (0.5 µg, 1.0 µg, 1.75 µg, 2.5 µg, and 3.0 µg) revealed an increased number of 50-kHz USVs with increasing doses. Friedman's ANOVA followed by Wilcoxon Test revealed that 3.0 µg of apomorphine significantly increased the emissions of flat 50-kHz USVs over 0.5 µg of apomorphine (χ^2 [4, 15] = 27. 808, $p < .001$; $Z = -2.975$, $p <. 003$; Figure 6). No dose of apomorphine was able to significantly increase the number of frequency modulated (FM) vocalizations (χ^2 [4, 15] = 8. 065, $p > .080$; Figure 6). Since increasing doses of apomorphine could not increase FM vocalizations, in the remainder of the thesis 50-kHz USVs will not be divided into flat or FM vocalizations but counted jointly.

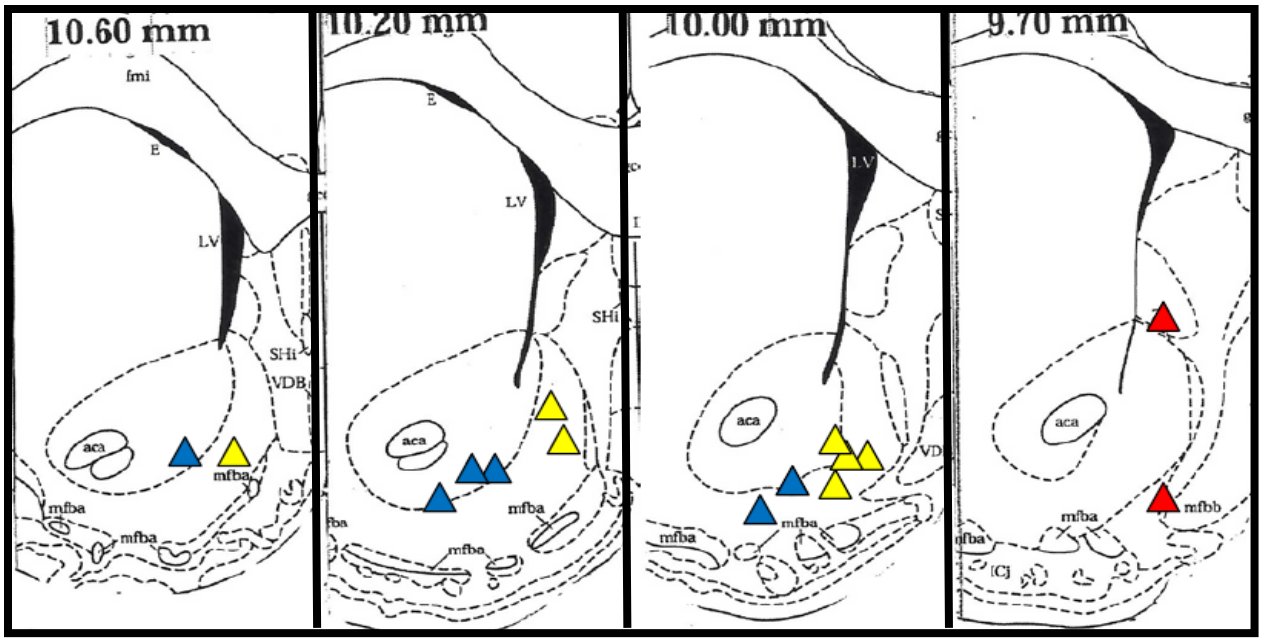

Figure 5: Localization for dose-response experiment in the nucleus accumbens: Mapping of injection sites for 0.5µg, 1.0 µg, 1.5 µg, 1.75 µg and 3.0 µg of apomorphine. Red triangles showed no recorded vocalizations, blue triangles represent injection sites that initiated calls between 1-10 50-kHz USVs, and the yellow triangles represent injection sites that initiated calls between 11-20 50-kHz USVs. All rats were injected with all doses of apomorphine and saline. Numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. Some abbreviations: aca – anterior commusure; mfb – medial forebrain bundle; SHi – septohippocampal nucleus; VDB; vertical limb of the diagonal band; LV – lateral ventricle. Numbers represent distance from lambda.

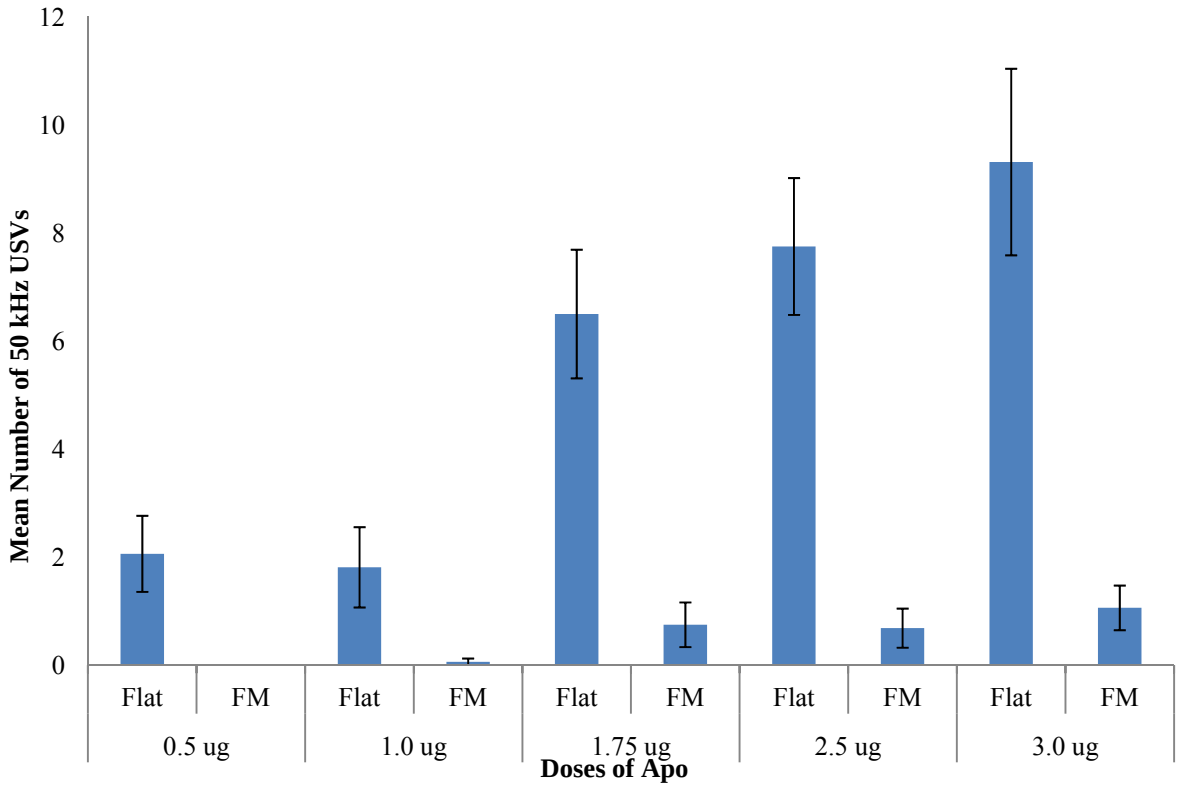

Figure 6: Dose-response relationship between apomorphine dose and number of 50-kHz USVs produced. Apomorphine (Apo) delivered to the medial shell of the nucleus accumbens was able to increase the number of flat 50-kHz USVs but was unable to significantly increase frequency modulated (FM) 50-kHz USVs. The largest quantity of 50-kHz USVs was induced by 3.0 µg of apomorphine. Error bars represent the S.E.M.

Injection group 1 a: Can injection of apomorphine into the medial shell of the nucleus accumbens, prior to injection of carbachol into the AH-MPO, decrease the number of emitted 22-kHz USVs?

22-kHz USVs: Localization of injection sites for injection group 1a are displayed in Figure 7 and 8. Injection of apomorphine into the medial shell of the nucleus accumbens one minute prior to injection of carbachol into the AH-MPO (apo+carb) significantly reduced the number of emitted 22-kHz USVs compared to USVs emitted after the saline-carbachol (sal+carb) injection (χ^2 [3, 11] = 33. 000, $p < .001$; Z = -2.934, $p < .001$; Figure 9). Analysis of call parameters did show significant differences. Injection of apomorphine into the medial shell of the nucleus accumbens before injection of carbachol into the AH-MPO significantly reduced the duration of 22-kHz USVs (χ^2 [3, 56] = 151.76, $p < .001$; Z = -3.997, $p < .001$; Figure 10). Apomorphine was unable to alter the peak frequency of 22-kHz USVs when compared to sal+carb injection conditions (χ^2 [3, 56] = 164.4, $p < .001$; Z = -1.313, p = .130; Figure 11).

50-kHz USVs: Injection of apomorphine into the shell of the nucleus accumbens and saline into the AH-MPO (apo+sal) significantly increased the number of 50-kHz USVs over sal+carb (χ^2 [3, 11] = 21. 835, p =. 000; Z = -2. 851, p = .004; Figure 12) conditions. Injection of apomorphine and carbachol (apo+carb) significantly attenuated the emission of 50-kHz USVs compared to apomorphine saline injections (apo+sal; Z = -2.941, p = .003; Figure 12). Injection of carbachol into the AH-MPO, after injection of apomorphine into the medial shell of the nucleus accumbens, was unable to alter the duration (χ^2 [3, 20] = .294 p = .961; Figure 13) or peak frequency (χ^2 [3, 20] = 2.700, p =.440; Figure 14) of 50-kHz USVs.

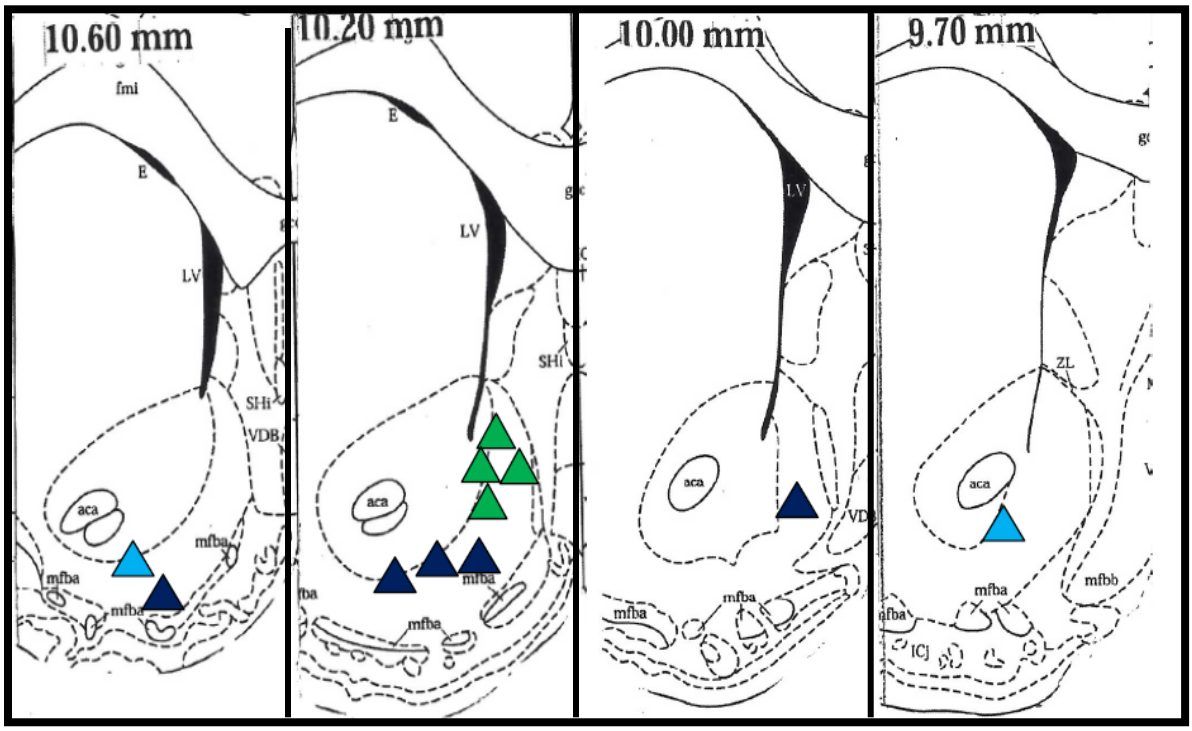

Figure 7: Mapping of injection sites (triangles) for 3.0 µg of apomorphine injected into the shell of the nucleus accumbens. The triangles are colour coded for the magnitude of the response. Blue triangles elicited a response between 1-10 50-kHz USVs, dark blue triangles elicited between 11-20 50-kHz USVs and the green triangles elicited between 21-30 50-kHz USVs. Numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. For abbreviations see Figure 5.

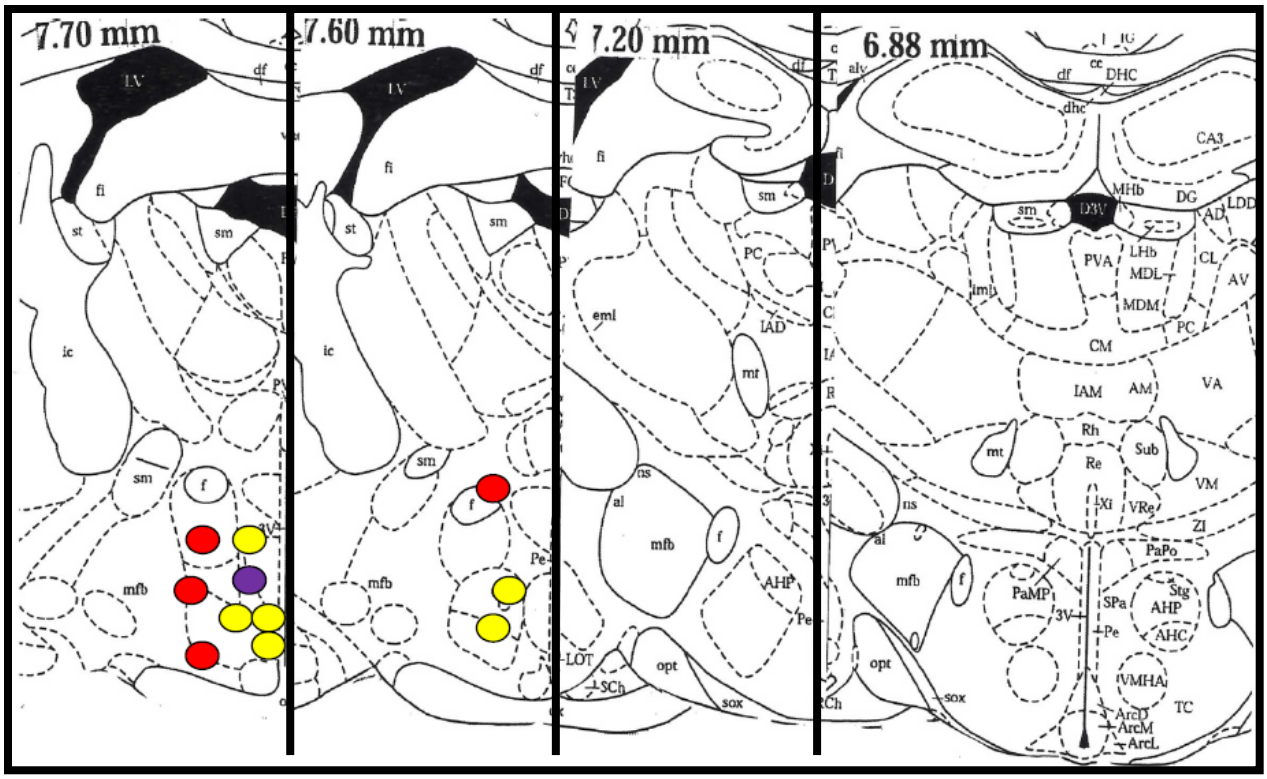

Figure 8: Mapping of injection sites (circles) for 1.0 µg of carbachol injected into the AH-MPO. The circles are colour coded for the magnitude of the response. Red circles elicited between 0-100 22-kHz USVs, yellow circles elicited between 101-200 22-kHz USVs and the purple circles elicited between 201-300 22-kHz USVs. Numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. Selected abbreviations: AHP- anterior hypothalamic area; sm – stria medullaris.

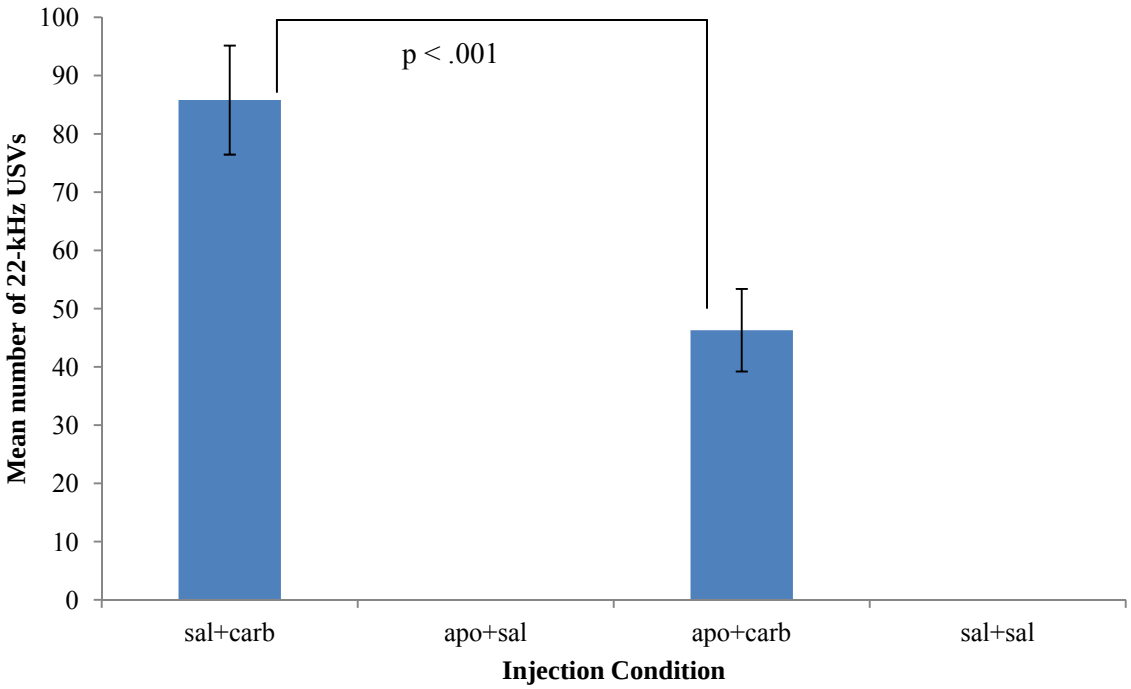

Figure 9: Mean number of 22-kHz USVs (± S.E.M.) recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean number of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the AH-MPO, was able to significantly reduce the mean number of recorded 22-kHz USVs. Injection conditions were as follows : sal+carb – saline injected into the sNa and carbachol injected into AH-MPO; apo+sal – apomorphine injected into the sNa and saline injected into the AH-MPO; apo+carb – apomorphine into the sNA and carbachol into AH-MPO; sal+sal – saline injected into the sNa and saline injected into the AH-MPO

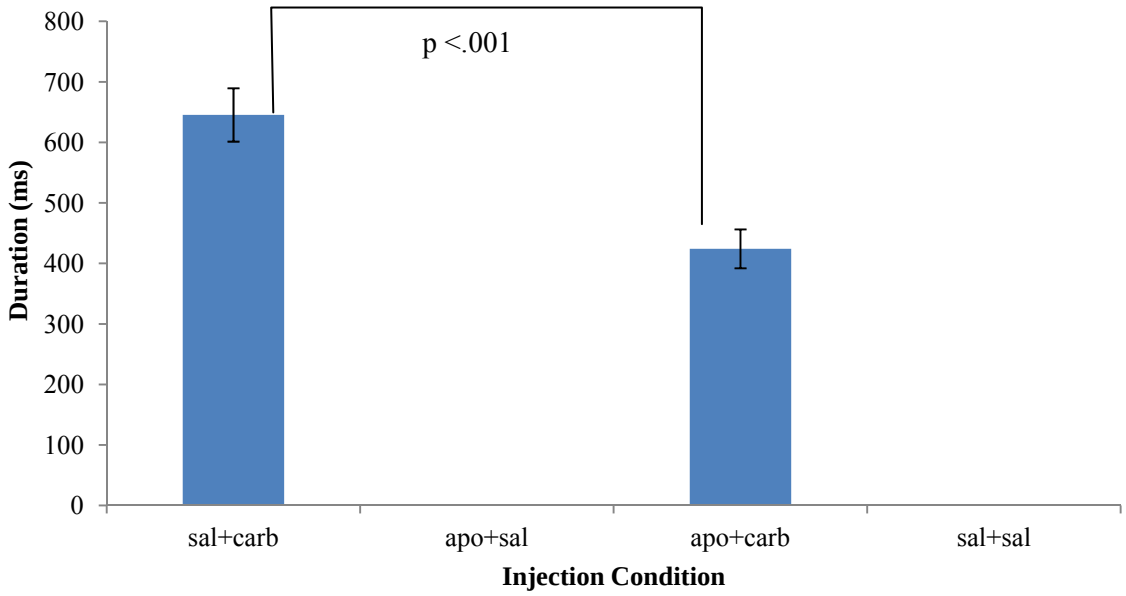

Figure 10: Mean duration (± S.E.M.) of recorded 22-kHz USVs. The x-axis represents the various injection conditions and the y-axis represents the mean duration of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the AH-MPO, was able to significantly alter the duration of the 22-kHz USVs. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into AH-MPO; apo+sal – apomorphine injected into the sNa and saline injected into the AH-MPO; apo+carb – apomorphine into the sNA and carbachol into AH-MPO; sal+sal – saline injected into the sNa and saline injected into the AH-MPO.

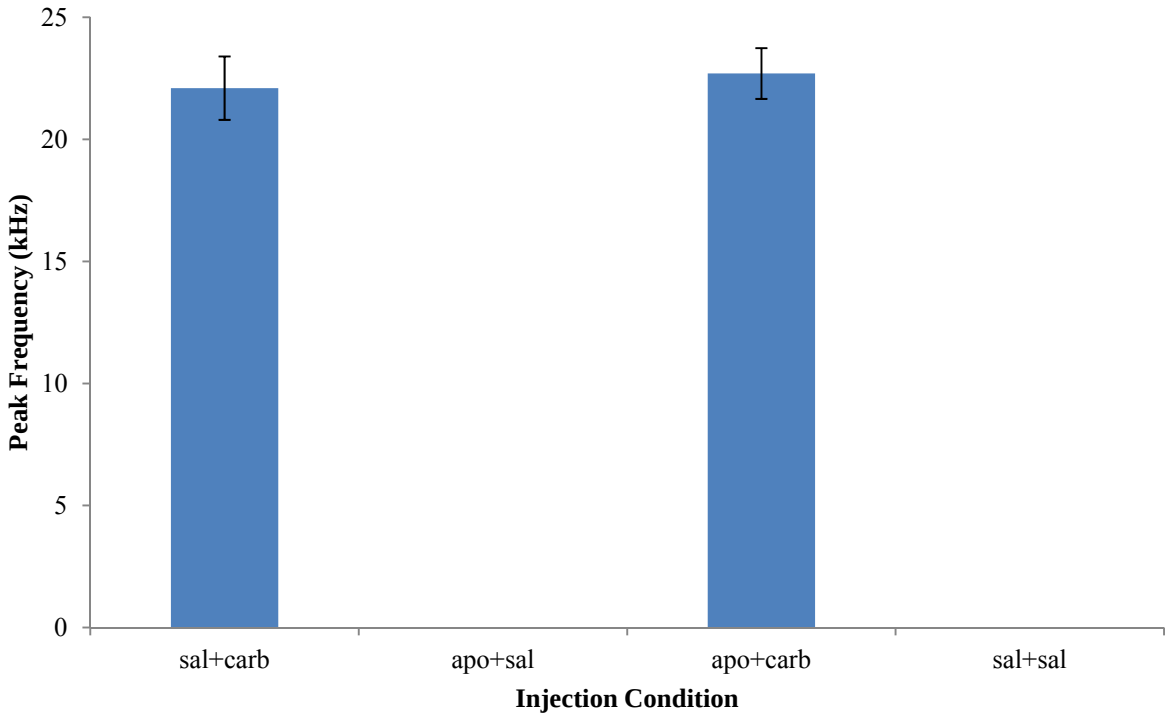

Figure 11: Mean peak frequency (± S.E.M.) of recorded 22-kHz USVs under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean peak frequency of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the AH-MPO, was unable to alter the peak frequency of the 22-kHz USVs. Injection conditions were as follows : sal+carb – saline injected into the sNa and carbachol injected into AH-MPO; apo+sal – apomorphine injected into the sNa and saline injected into the AH-MPO; apo+carb – apomorphine into the sNA and carbachol into AH-MPO; sal+sal – saline injected into the sNa and saline injected into the AH-MPO.

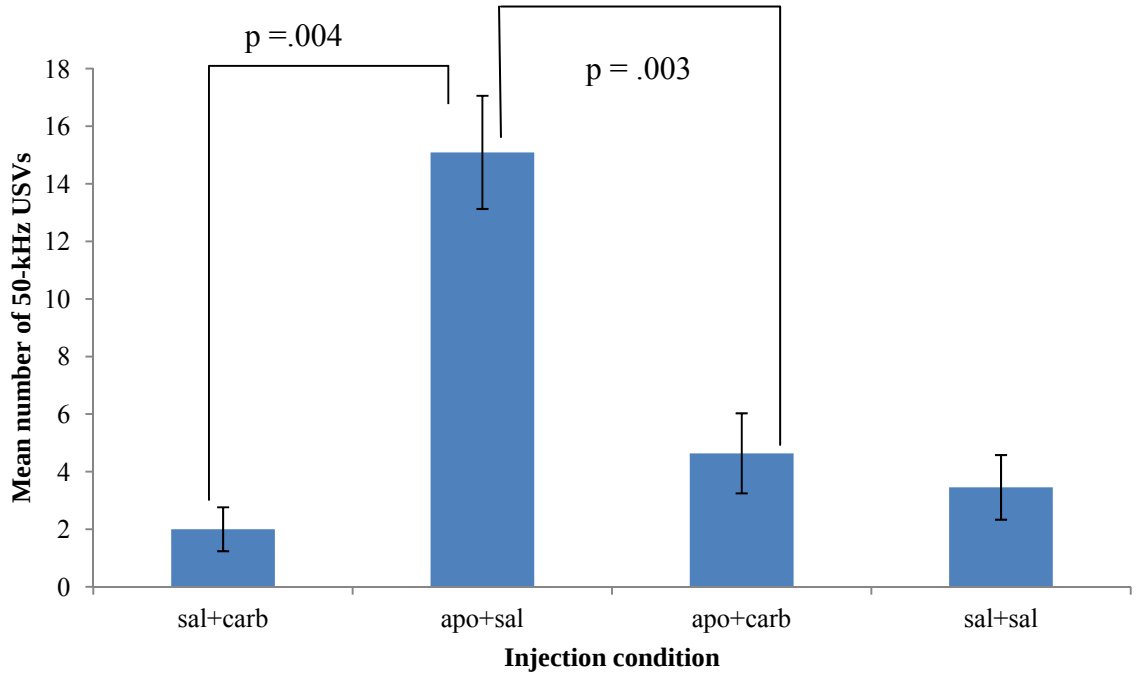

Figure 12: Mean number of 50-kHz USVs (± S.E.M.) recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean number of 50-kHz USVs emitted. Injection of carbachol (carb) into the AH-MPO, one minute after injection of apomorphine (apo) into the shell, was able to significantly reduce the mean number of 50-kHz USVs. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into AH-MPO; apo+sal – apomorphine injected into the sNa and saline injected into the AH-MPO; apo+carb – apomorphine into the sNA and carbachol into AH-MPO; sal+sal – saline injected into the sNa and saline injected into the AH-MPO.

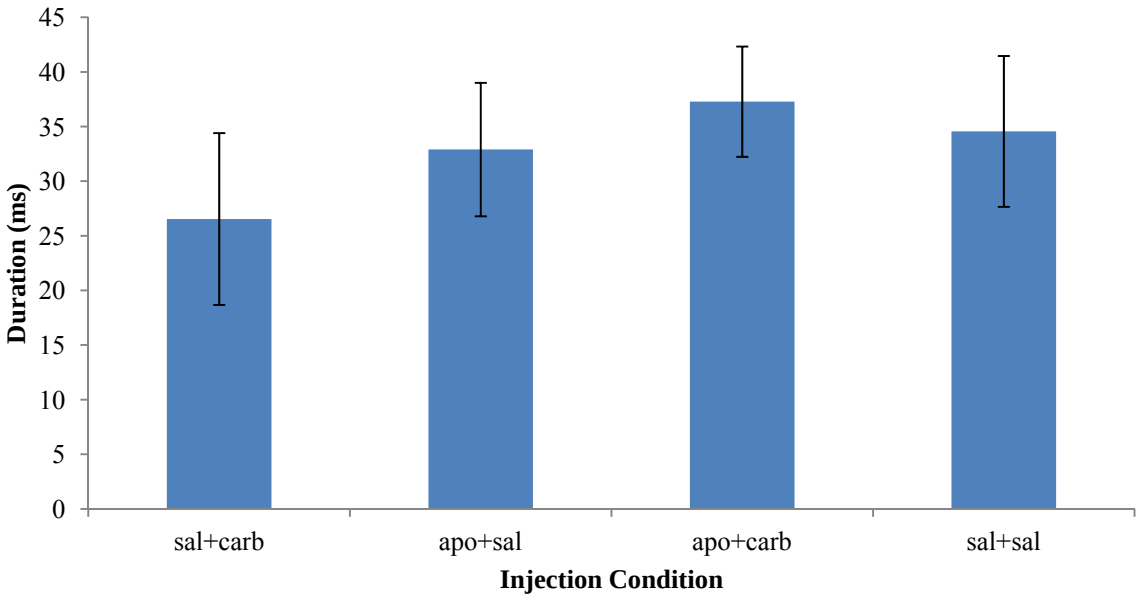

Figure 13: Mean duration of recorded 50-kHz USVs (±S. E. M.). The x-axis represents the various injection conditions and the y-axis represents the mean duration of 50-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the AH-MPO, was unable to significantly change the duration of 50-kHz USVs USVs. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into AH-MPO; apo+sal – apomorphine injected into the sNa and saline injected into the AH-MPO; apo+carb – apomorphine into the sNA and carbachol into AH-MPO; sal+sal – saline injected into the sNa and saline injected into the AH-MPO.

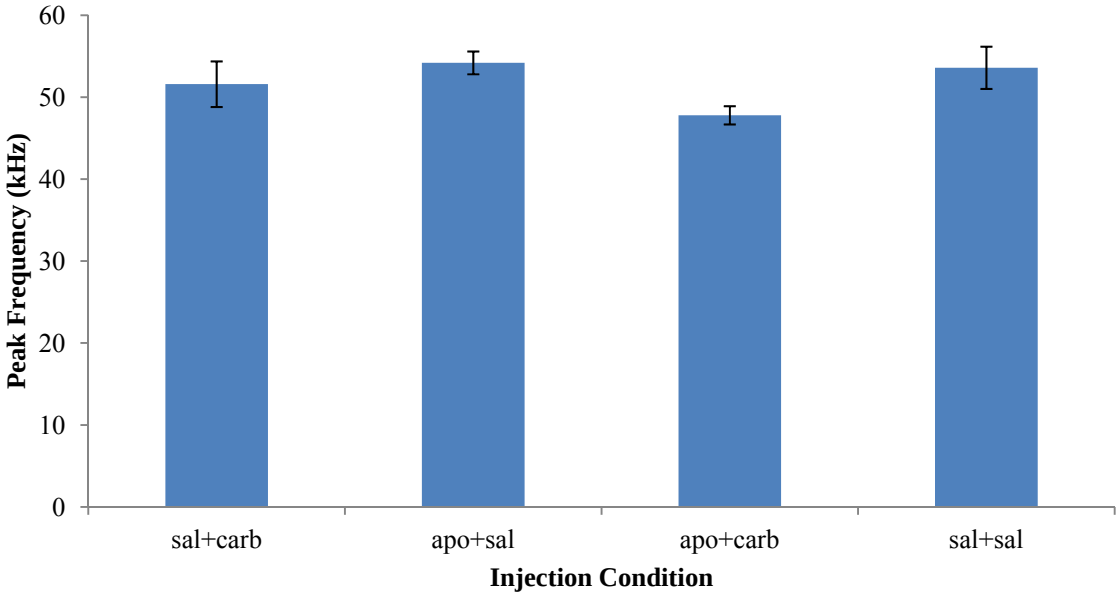

Figure 14: Mean peak frequency (±S. E. M.) of recorded 50-kHz USVs recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean value of peak frequency in kHz. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the AH-MPO, was unable to significantly change the mean peak frequency of 50-kHz USVs USVs. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into AH-MPO; apo+sal – apomorphine injected into the sNa and saline injected into the AH-MPO; apo+carb – apomorphine into the sNA and carbachol into AH-MPO; sal+sal – saline injected into the sNa and saline injected into the AH-MPO.

Injection set 1b: Can injection of apomorphine into the medial shell of the nucleus accumbens, prior to injection of carbachol into the LS, decrease the number of emitted 22-kHz USVs?

22-kHz USVS: Localization of injection sites for injection set 1b can be found in Figures 15 and 16. Injection of apomorphine into the medial shell of the nucleus accumbens one minute prior to injection of carbachol into the LS (apo+carb) significantly reduced the number of emitted 22-kHz USVs compared to the saline-carbachol (sal+carb) condition (χ^2 [3, 12] = 24.926, p = .000; Z= -2.075, p = .038; Figure 17). Analysis of call parameters also showed significant differences in the duration of 22-kHz USVs during the apo+carb condition compared to the sal+carb condition (χ^2 [3, 56] = 147. 3, p < .001; Z = -2. 304; p = .021; Figure 18) but the peak frequency of 22-kHz USVs remained unchanged (χ^2 [3, 56] = 151. 3, p < .001; Z = -1.44, p = .150; Figure 19).

50-kHz USVs: Apomorphine injected into the medial shell of the nucleus accumbens prior to saline injection into the LS (apo+sal) significantly increased the number of 50-kHz USVs compared to sal+sal injections (χ^2 [3, 12] = 20. 357, p =.000; Z = -2.688, p =.008; Figure 20) and saline-carbachol injections (Z = -2.312, p =.012; Figure 20). Injection of apomorphine into the medial shell of the nucleus accumbens, prior to injection of carbachol into the LS, significantly reduced the number of emitted 50-kHz USVs when compared to apo+sal injection (Z = -2.429, p = .015; Figure 20). Injection of carbachol into the LS, after injection of apomorphine into the medial shell of the nucleus accumbens, was unable to alter the duration (χ^2 [3, 20] = 1.275, p >.05; Figure 21) or the peak frequency (χ^2 [3, 20] =2.508, p >.05; Figure 22) of recorded 50-kHz USVs.

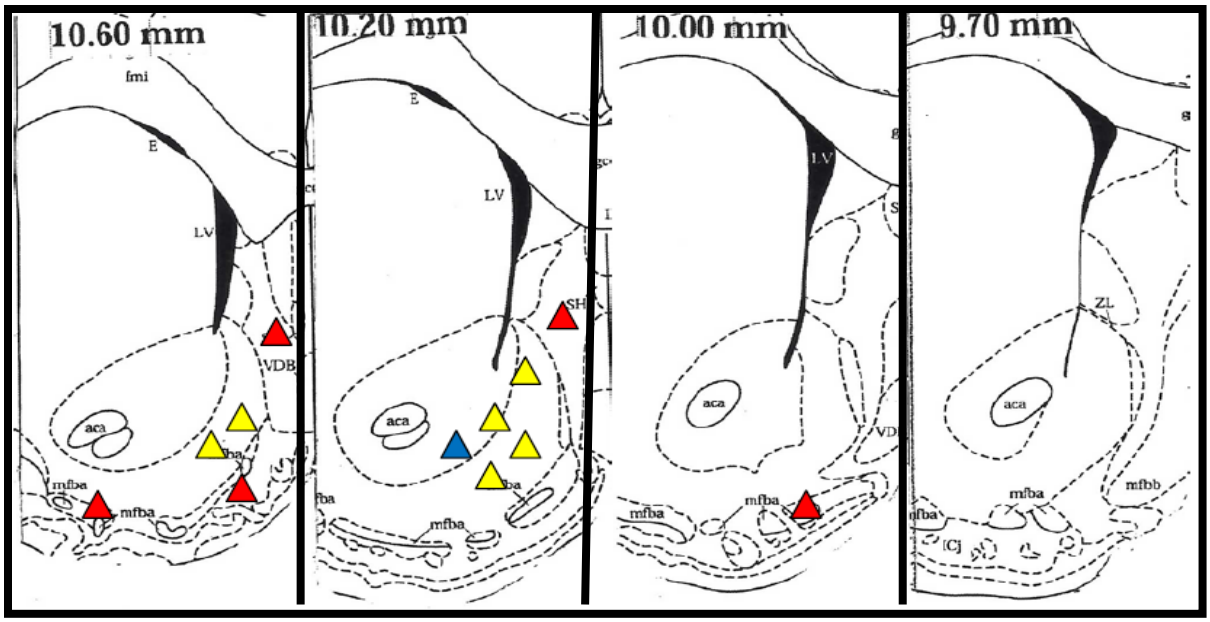

Figure 15: Mapping of injection sites (triangles) for 3.0 µg of apomorphine injected into the nucleus accumbens shell. The triangles are colour coded for the magnitude of the response. Red triangles elicited a response between 1-10 50-kHz USVs, the dark blue triangle elicited between 11-20 50-kHz USVs and the yellow triangles elicited between 21-30 50-kHz USVs. Numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. For abbreviations see Figure 5.

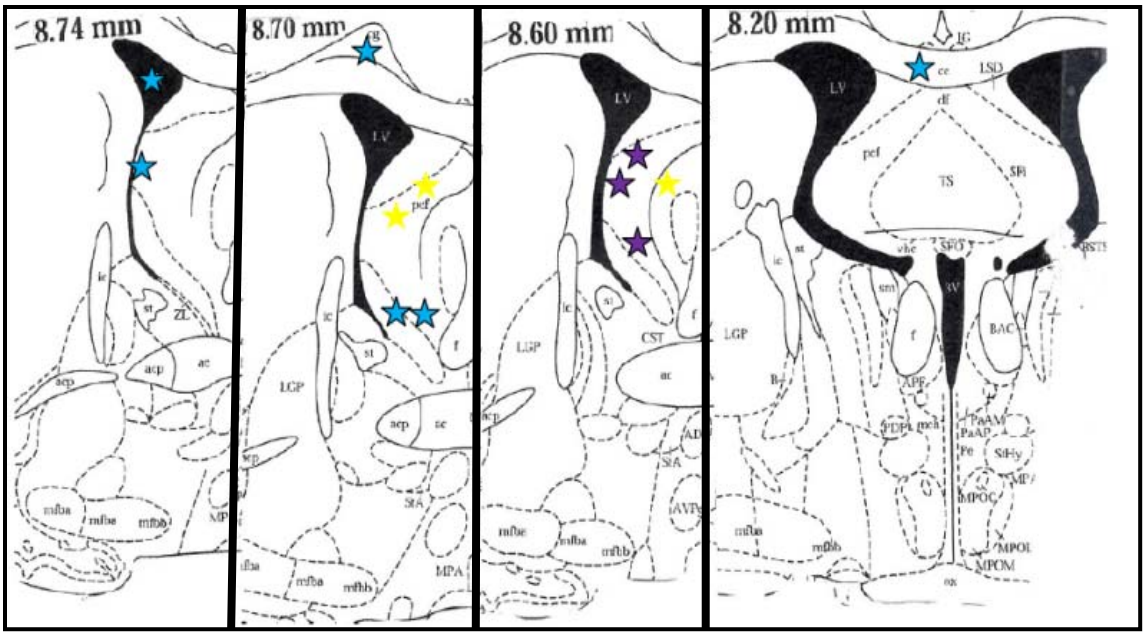

Figure 16: Mapping of injection sites for 1.0 µg of carbachol from the lateral septum. The stars are colour coded for the magnitude of the response. Blue stars elicited 0 – 10 22-kHz USVs, yellow stars elicited 101-200 USVs and purple stars were able to elicit between 201-300 22-kHz USVs. Numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. Selected abbreviations: LV = Lateral Ventricle; LS = Lateral Septum; cc = corpus callosum; f- fornix.

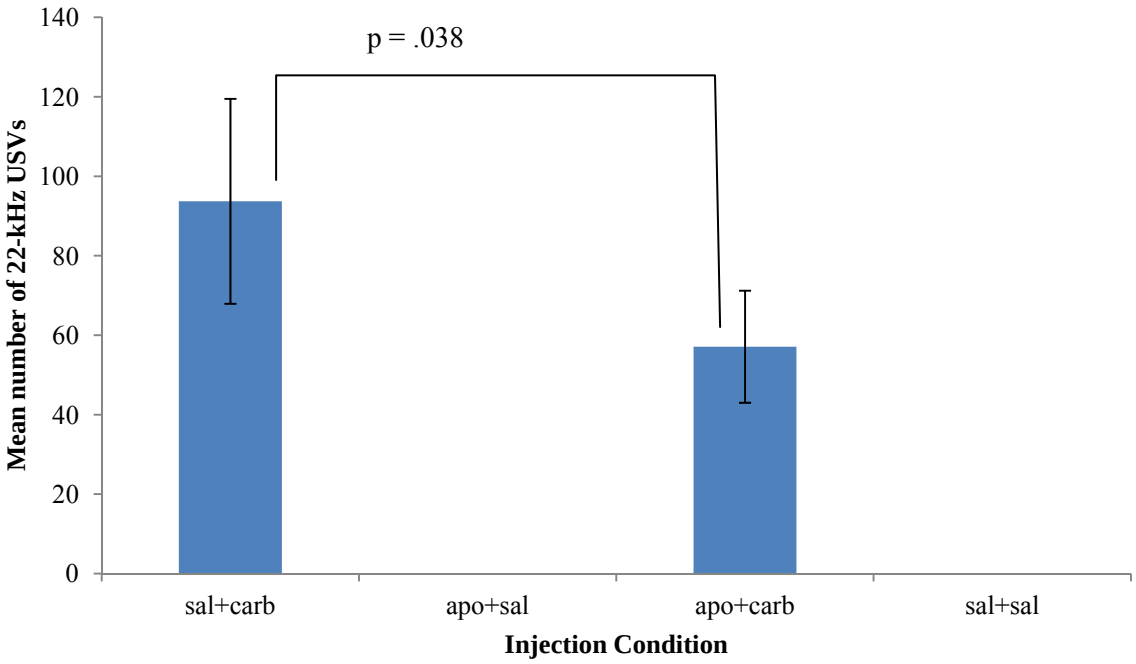

Figure 17: Mean number of 22-kHz USVs (± S.E.M.) recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean number of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the LS, was able to significantly reduce the mean number of recorded 22-kHz USVs as compared to the sal+carb condition. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into LS; apo+sal – apomorphine injected into the sNa and saline injected into the LS; apo+carb – apomorphine into the sNA and carbachol into LS; sal+sal – saline injected into the sNa and saline injected into the LS.

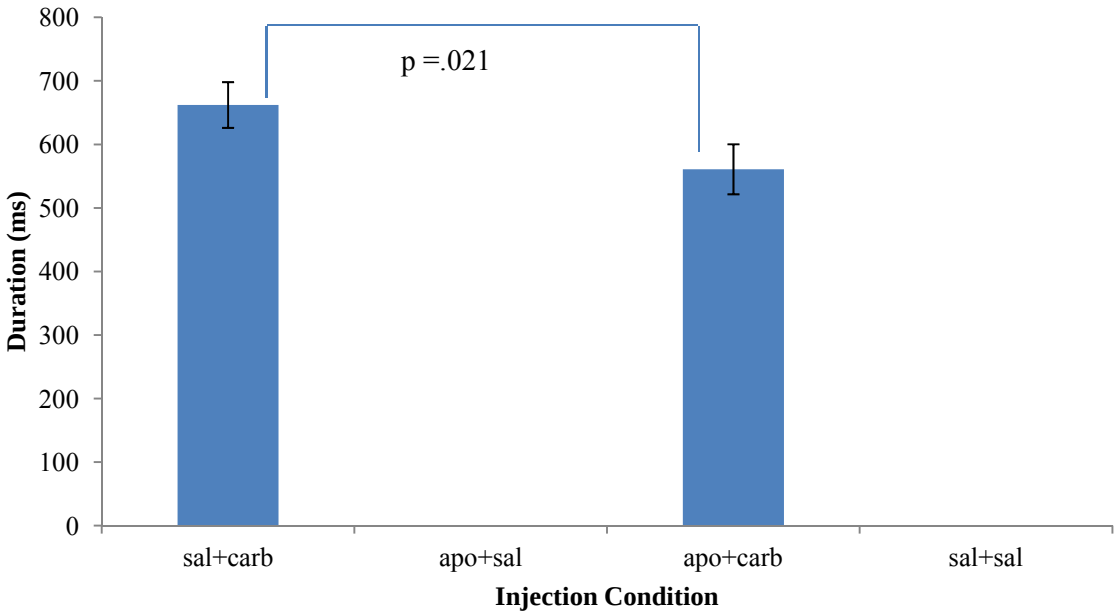

Figure 18: Mean duration (± S. E.M.) of the recorded 22-kHz USVs. The x-axis represents the various injection conditions and the y-axis represents the mean duration (ms) of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the LS, was able to significantly alter the duration (ms) of the 22-kHz USVs. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into LS; apo+sal – apomorphine injected into the sNa and saline injected into the LS; apo+carb – apomorphine into the sNA and carbachol into LS; sal+sal – saline injected into the sNa and saline injected into the LS.

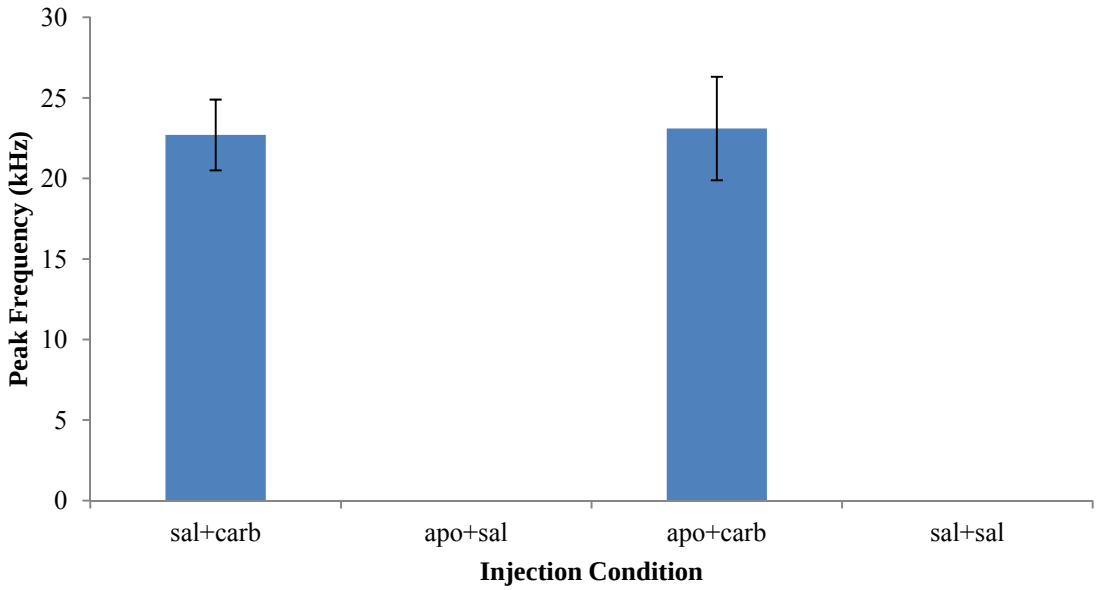

Figure 19: Mean peak frequency of 22-kHz USVs (± S.E.M.) under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean peak frequency of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the LS, was unable to alter the peak frequency of the 22-kHz USVs. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into LS; apo+sal – apomorphine injected into the sNa and saline injected into the LS; apo+carb – apomorphine into the sNA and carbachol into LS; sal+sal – saline injected into the sNa and saline injected into the LS.

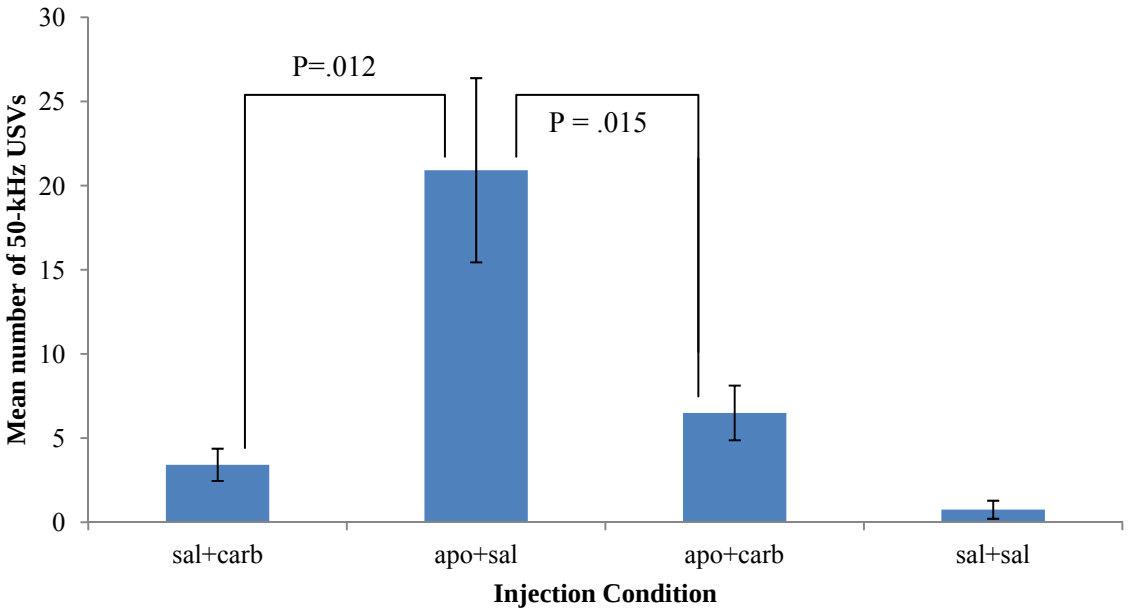

Figure 20: Mean number of 50-kHz USVs (± S.E.M.) recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean number of 50-kHz USVs emitted. Apo+sal conditions showed a significant increase in emission of 50-kHz USVs compared to sal+sal and sal+carb conditions. Injection of carbachol (carb), one minute after an injection of apomorphine (apo) into the shell of the accumbens significantly reduced the mean number of 50-kHz USVs. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into LS; apo+sal – apomorphine injected into the sNa and saline injected into the LS; apo+carb – apomorphine into the sNA and carbachol into LS; sal+sal – saline injected into the sNa and saline injected into the LS.

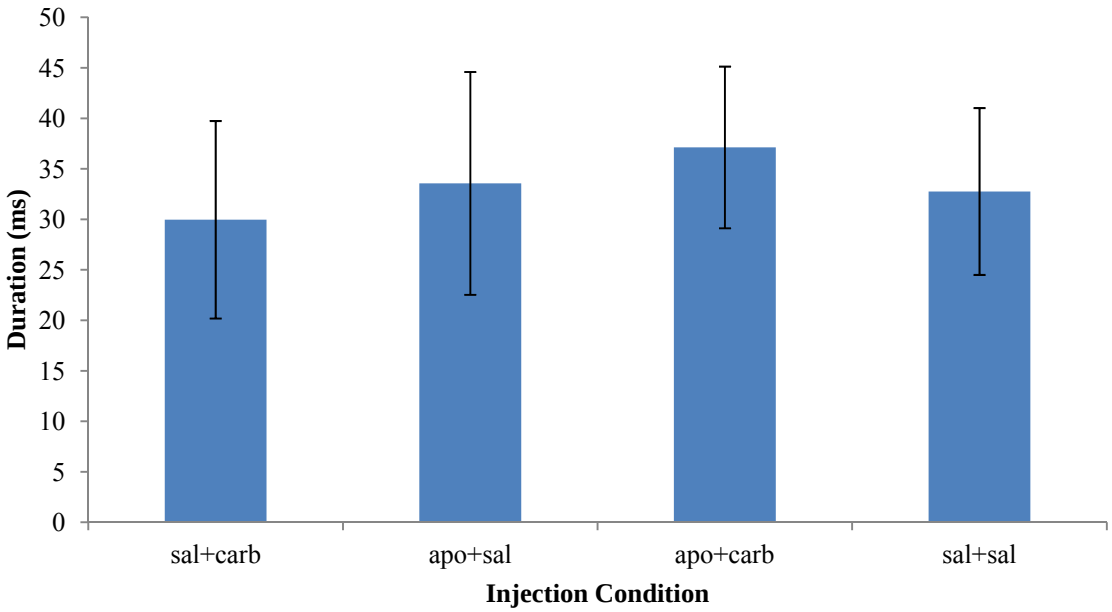

Figure 21: Mean duration (ms) of 50-kHz USVs (±S.E.M.). The x-axis represents the various injection conditions and the y-axis represents the mean duration of 50-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the LS, was unable to significantly change the duration of 50-kHz USVs. Calls in all conditions had comparable durations. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into LS; apo+sal – apomorphine injected into the sNa and saline injected into the LS; apo+carb – apomorphine into the sNA and carbachol into LS; sal+sal – saline injected into the sNa and saline injected into the LS.

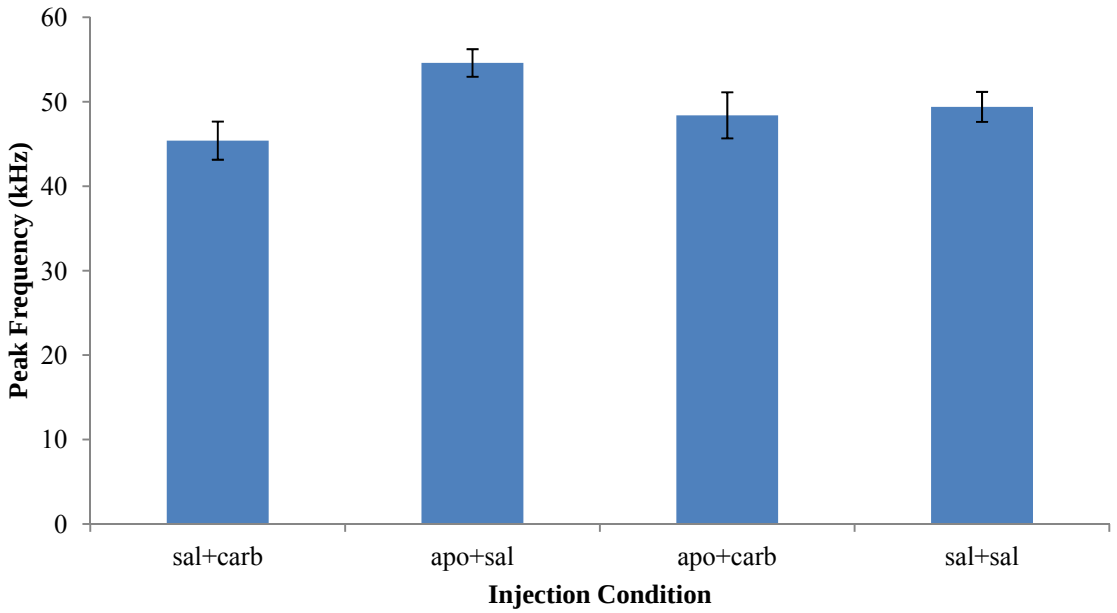

Figure 22: Mean peak frequency (± S.E.M.) of 50-kHz USVs under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean peak frequency of 50-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the LS, was unable to significantly change the mean peak frequency of 50-kHz USVs. Calls in all conditions had comparable peak frequencies. Injection conditions were as follows: sal+carb – saline injected into the sNa and carbachol injected into LS; apo+sal – apomorphine injected into the sNa and saline injected into the LS; apo+carb – apomorphine into the sNA and carbachol into LS; sal+sal – saline injected into the sNa and saline injected into the LS.

Injection set 2a: Can carbachol injected into the AH-MPO, prior to apomorphine injection into the shell of the nucleus accumbens, decrease the number of emitted 50-kHz USVs?

50-kHz USVS: Localizations for this injection set can be found in Figure 23 and 24. Injection of carbachol into the AH-MPO one minute prior to injection of apomorphine into the medial shell of the nucleus accumbens was unable to significantly reduce the number of recorded 50-kHz USVs (χ^2 [3, 12] = 12.207, $p < .005$; $Z = -1.844$, $p = .066$; Figure 25). The sal+apo condition was able to significantly increase the number of emitted 50-kHz USVs compared to sal+sal injection ($Z = - 2.201$, $p = .021$; Figure 25) while the carb+apo condition showed no significant difference in facilitating the production of 50-kHz USVs compared to sal+sal condition ($Z = -1. 841$, $p = .198$; Figure 25). The differential USV responses that the sal+apo and carb+apo injection conditions produced suggests that injection of carbachol into the AH-MPO showed a trend in reducing the number of apomorphine induced 50-kHz USVs. Injection of carbachol into the AH-MPO prior to injection of apomorphine was unable to alter both the duration (χ^2 [3, 20] = 3.34, $p > .05$; Figure 26) and peak frequency (χ^2 [3, 20] = 2.201, $p >. 05$; Figure 27) of recorded 50-kHz USVs.

22-kHz USVS: Injection of carbachol followed by saline induced a high number of 22-kHz USVs (Figure 28). Injection of apomorphine into the medial shell of the nucleus accumbens after carbachol was able to reduce the mean number of recorded 22-kHz USVs (carb+apo) compared to carbachol-saline injections (χ^2 [3, 12] = 17.118; $p = .001$; $Z = -2.201$, $p = .038$; Figure 28). Analysis of recorded 22-kHz USVs showed that apomorphine was also able to reduce the mean duration of 22-kHz USVs compared to controls (carb+sal) injections (χ^2 [3, 56] = 156.172, $p < .001$; $Z = -5.422$, $p < .001$; Figure 29) but was unable to alter the peak frequency of recorded 22-kHz USVs (χ^2 [3, 56] = 147. 331, $p < .001$; $Z = .216$, $p > .05$; Figure 30).

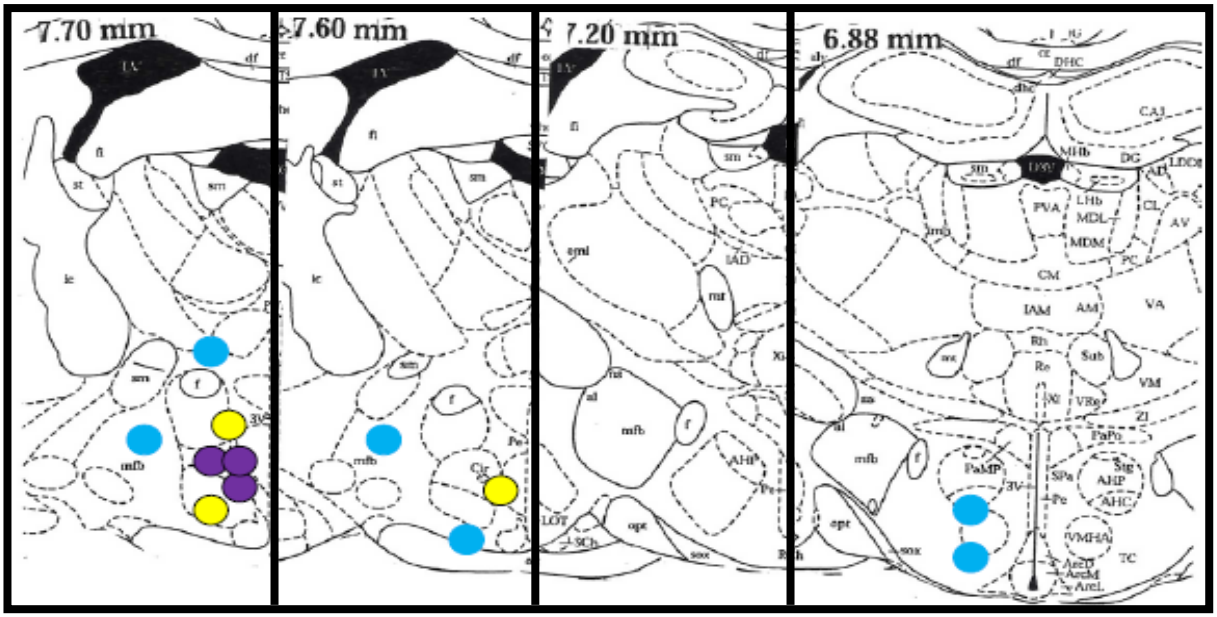

Figure 23: Mapping of injection sites (circles) for 1.0 µg of carbachol in the AH-MPO. The circle localizations are colour coded for the number of the 22-kHz USVs. Blue circles were able to elicit between 0-100 22-kHz USVs, yellow circles were able to elicit between 100-200 22-kHz USVs and purple circles were able to elicit between 201-300 22-kHz USVs. The numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. For selected abbreviations see Figure 8.

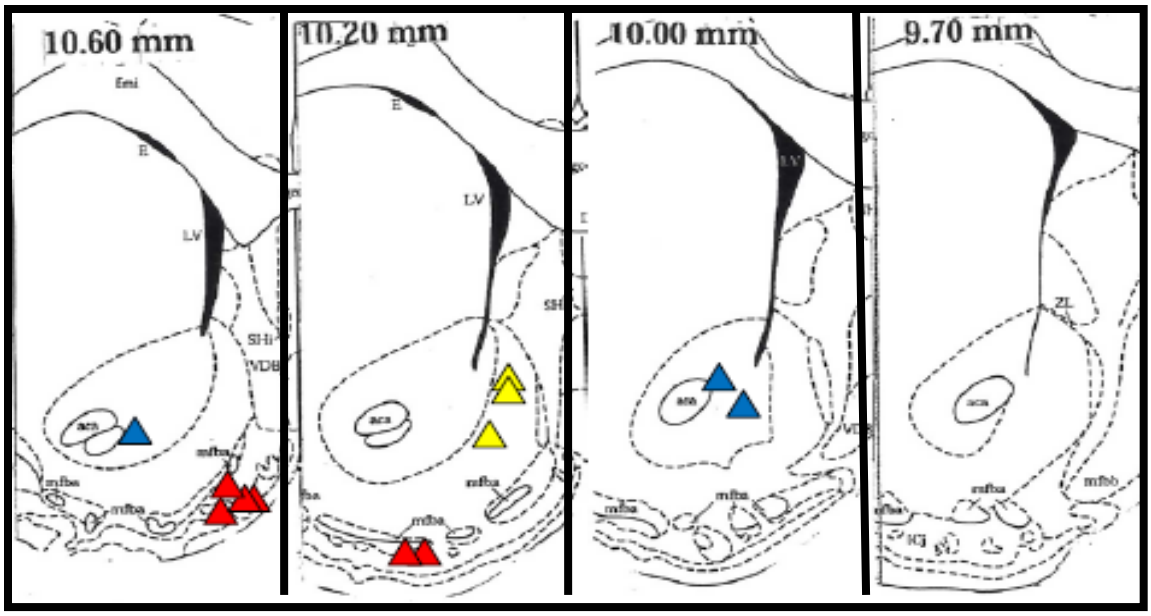

Figure 24: Mapping of localization sites (triangles) for 3.0 µg of apomorphine injected into the shell of the nucleus accumbens. The localization triangles are colour coded for the magnitude of the response. Red triangles were able to induce 1-10 50-kHz USVs, blue triangles were able to induce 11-20 50-kHz USVs and yellow triangles were able to elicit 21-45 50-kHz USVs. The numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. For selected abbreviations see Figure 1.

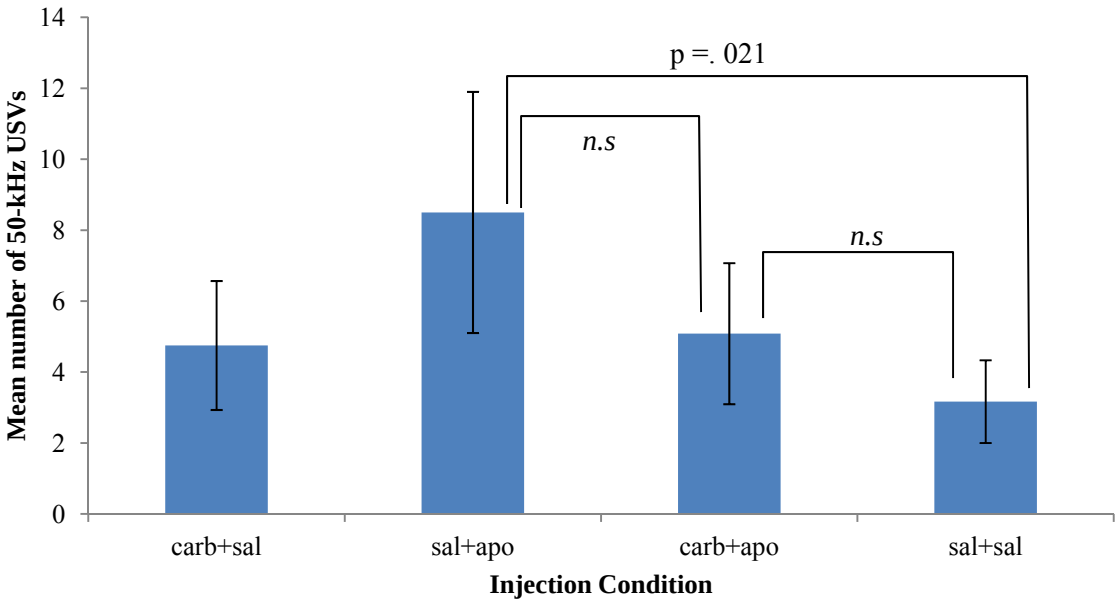

Figure 25: Mean number of 50-kHz USVs (± S.E.M.) recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean number of 50-kHz USVs emitted. Injection of carbachol (carb) into the AH-MPO, one minute before an injection of apomorphine (apo) into the nucleus accumbens, significantly reduced the mean number of recorded 50-kHz USVs. Injection conditions were as follows: carb+sal – carbachol was injected into the AH-MPO and saline was injected into the medial shell of the nucleus accumbens; sal+apo – saline was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the AH-MPO and saline was injected into the medial shell of the nucleus accumbens.

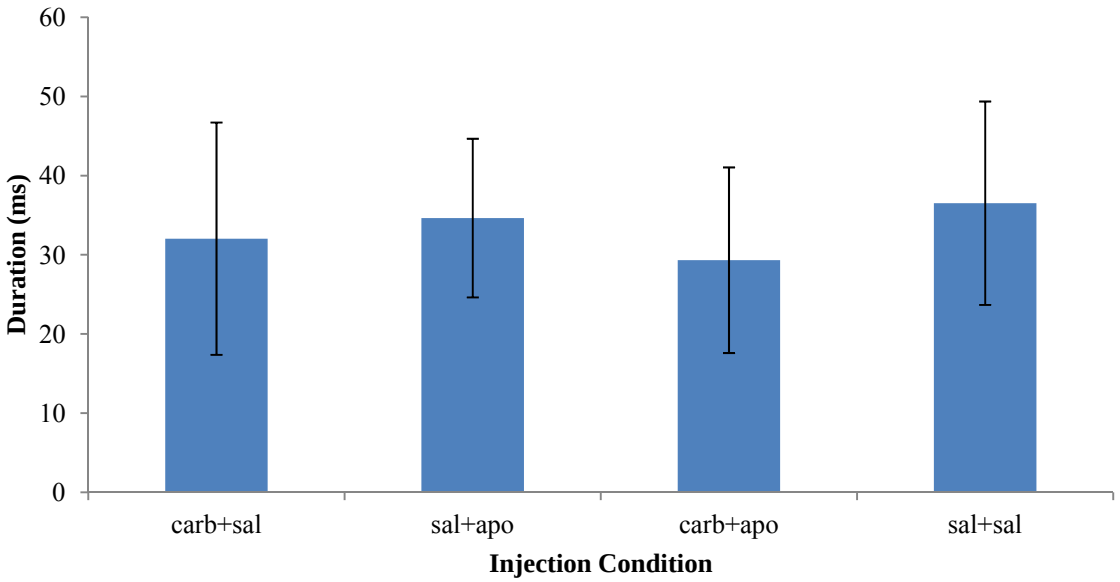

Figure 26: Mean duration (± S.E.M.) of 50-kHz USVs. The x-axis represents the various injection conditions and the y-axis represents the mean duration of 50-kHz USVs emitted. Injection of apomorphine into the medial shell of the nucleus accumbens, one minute after an injection of carbachol into the AH-MPO, was unable to significantly change the duration of 50-kHz USVs USVs Injection conditions were as follows: carb+sal – carbachol was injected into the AH-MPO and saline was injected into the medial shell o the nucleus accumbens; sal+apo – saline was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the AH-MPO and saline was injected into the medial shell of the nucleus accumbens.

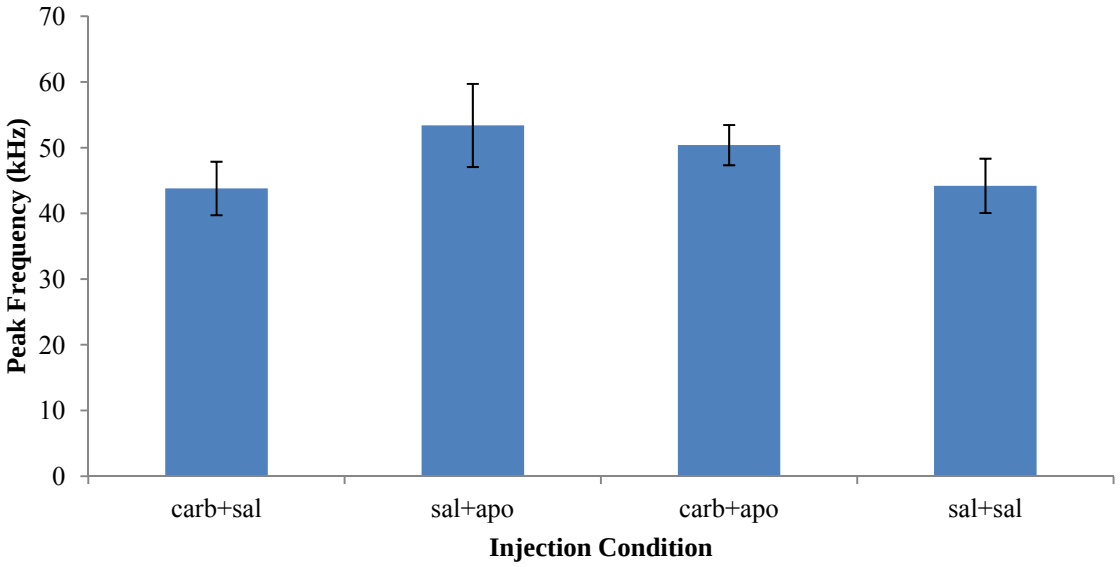

Figure 27: Mean peak frequency (± S.E.M.) of 50-kHz USVs under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean peak frequency of 50-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute after an injection of carbachol (carb) into the AH-MPO, was unable to significantly change the mean peak frequency of 50-kHz USVs USVs Injection conditions were as follows: carb+sal – carbachol was injected into the AH-MPO and saline was injected into the medial shell o the nucleus accumbens; sal+apo – saline was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the AH-MPO and saline was injected into the medial shell of the nucleus accumbens.

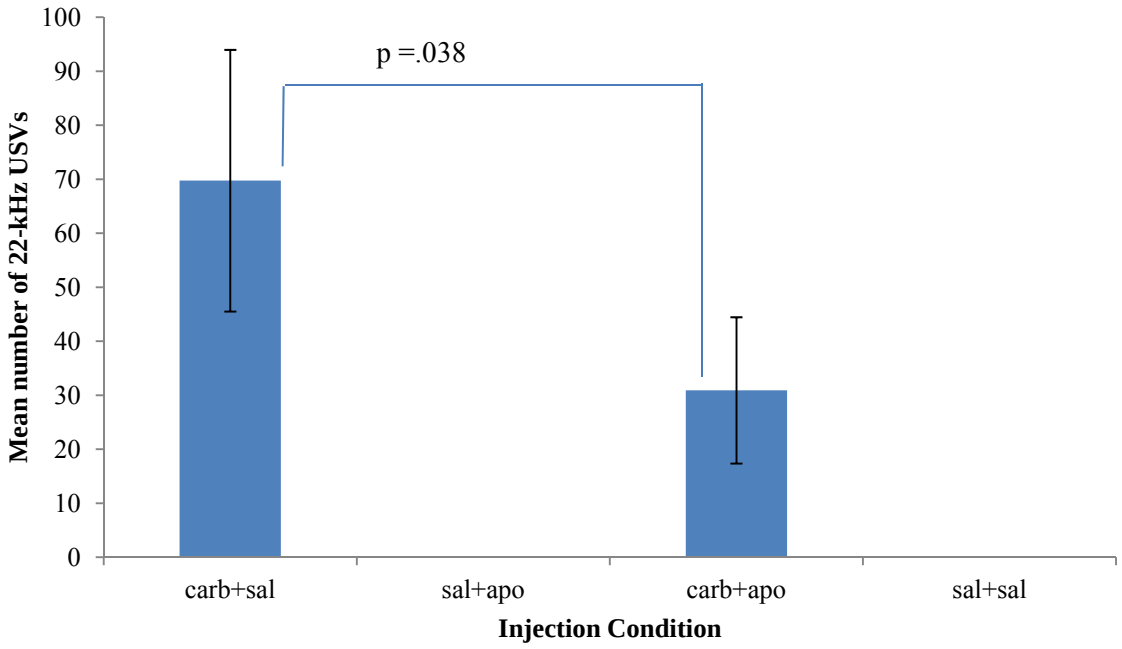

Figure 28: Mean number of 22-kHz USVs (± S.E.M.) recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean number of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute after an injection of carbachol (carb) into the AH-MPO, was able to significantly reduce the mean number of recorded 22-kHz USVs. Injection conditions were as follows: carb+sal – carbachol was injected into the AH-MPO and saline was injected into the medial shell o the nucleus accumbens; sal+apo – saline was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the AH-MPO and saline was injected into the medial shell of the nucleus accumbens.

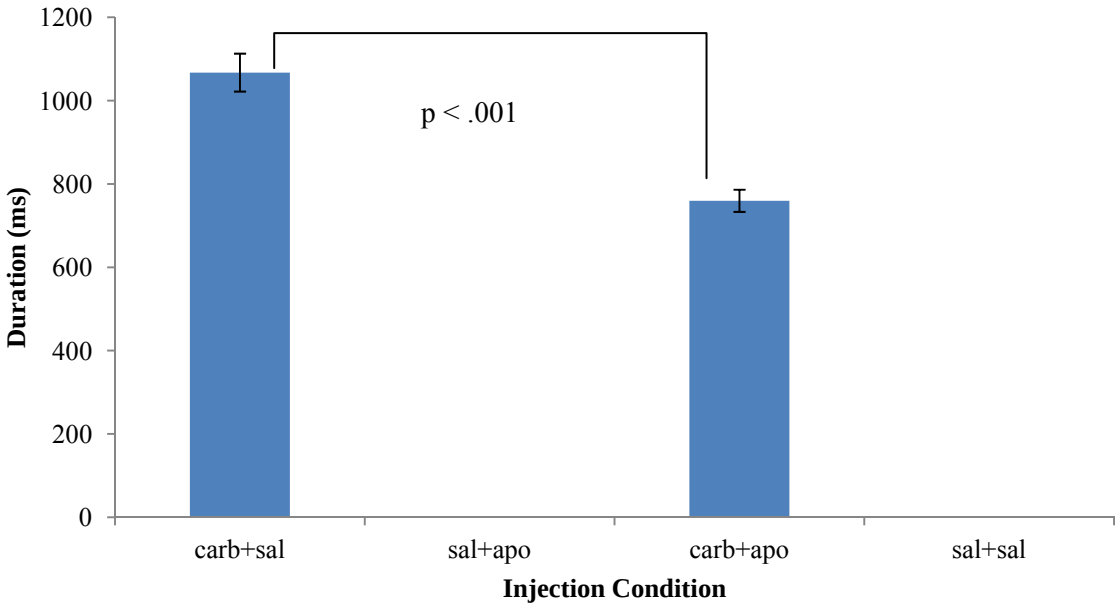

Figure 29: Mean duration (±S.E.M.) of the same 22-kHz USVs in figure 22. The x-axis represents the various injection conditions and the y-axis represents the mean duration of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute prior to injection of carbachol (carb) into the AH-MPO, was able to significantly alter the duration of the 22-kHz USVs. Injection conditions were as follows: carb+sal – carbachol was injected into the AH-MPO and saline was injected into the medial shell o the nucleus accumbens; sal+apo – saline was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the AH-MPO and saline was injected into the medial shell of the nucleus accumbens.

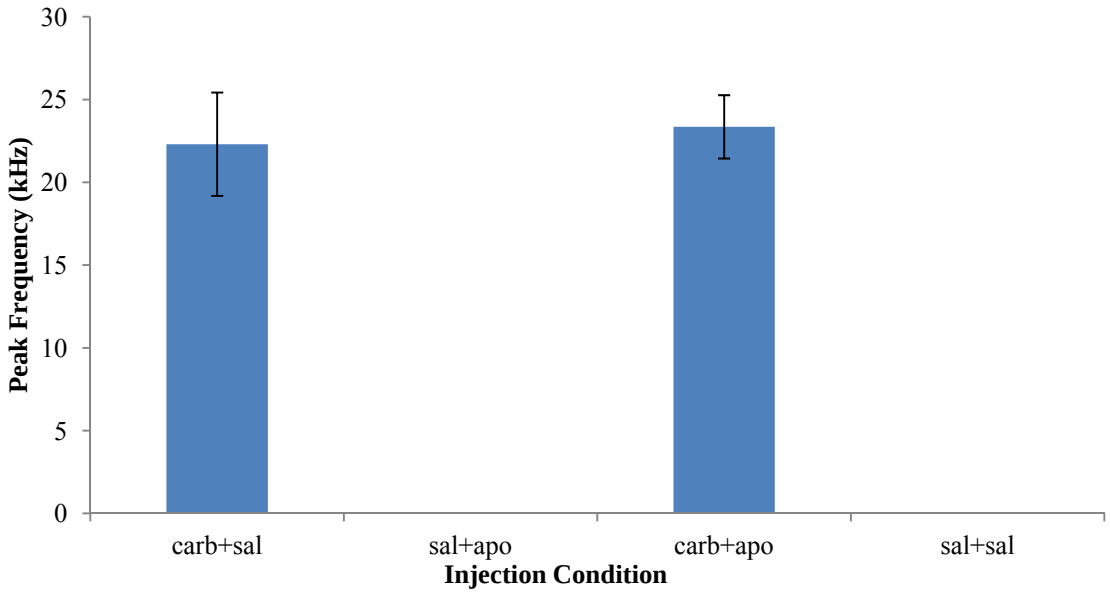

Figure 30: Mean peak frequency (±S.E.M.) of 22-kHz USVs under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean peak frequency of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute after an injection of carbachol (carb) into the AH-MPO, was unable to alter the peak frequency of the 22-kHz USVs. Injection conditions were as follows: carb+sal – carbachol was injected into the AH-MPO and saline was injected into the medial shell o the nucleus accumbens; sal+apo – saline was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the AH-MPO and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the AH-MPO and saline was injected into the medial shell of the nucleus accumbens.

Injection set 2b: Can carbachol injected into the LS, prior to injection of apomorphine into the medial shell of the nucleus accumbens, decrease the number of emitted 50-kHz USVs?

50-kHz USVS: Localization of injections for injection set 2b can be found in Figures 31 and 32. Injection of carbachol into the LS one minute before injection of apomorphine into the medial shell of the nucleus accumbens (carb+apo) significantly reduced the mean number of emitted 50-kHz USVs compared to the saline-apomorphine (sal+apo) condition (χ^2 [3, 10] = 13.500, p = .004; Z = -2.205, p = .028; Figure 33). The carbachol-apomorphine (carb+apo) was not significantly different from the saline-saline condition to in production of 50-kHz USVs (Z = 1.620, p > .05; Figure 33). Injection of carbachol prior to injection of apomorphine was unable to alter the duration (χ^2 [3, 20] = 1.708, p = .463; Figure 34) or the peak frequency (χ^2 = [3, 20] = 2. 563, p = .653; Figure 35) of 50-kHz USVs.

22-kHz USVS: Injection of carbachol into the LS followed by saline injection into the shell of the nucleus accumbens induced a high number of 22-kHz calls (Figure 36). Injection of apomorphine into the medial shell of the nucleus accumbens after carbachol injection into the LS significantly reduced the mean number of 22-kHz USVs compared to carb+sal injection condition (χ^2 [3, 10] = 22.833, p < .001; Z = -2.032, p = .042; Figure 36). Analysis of 22-kHz USVs showed that injection of apomorphine into the medial shell of the nucleus accumbens, after carbachol was injected into the LS, significantly reduced the duration of emitted 22-kHz USVs (χ^2 [3, 56] = 158.9, p = .000; Z = -5.672, p < .001; Figure 37) but did not change the peak frequency (χ^2 [3, 56] = 132.08, p = .001; Z = -1.160, p = .246; Figure 38)

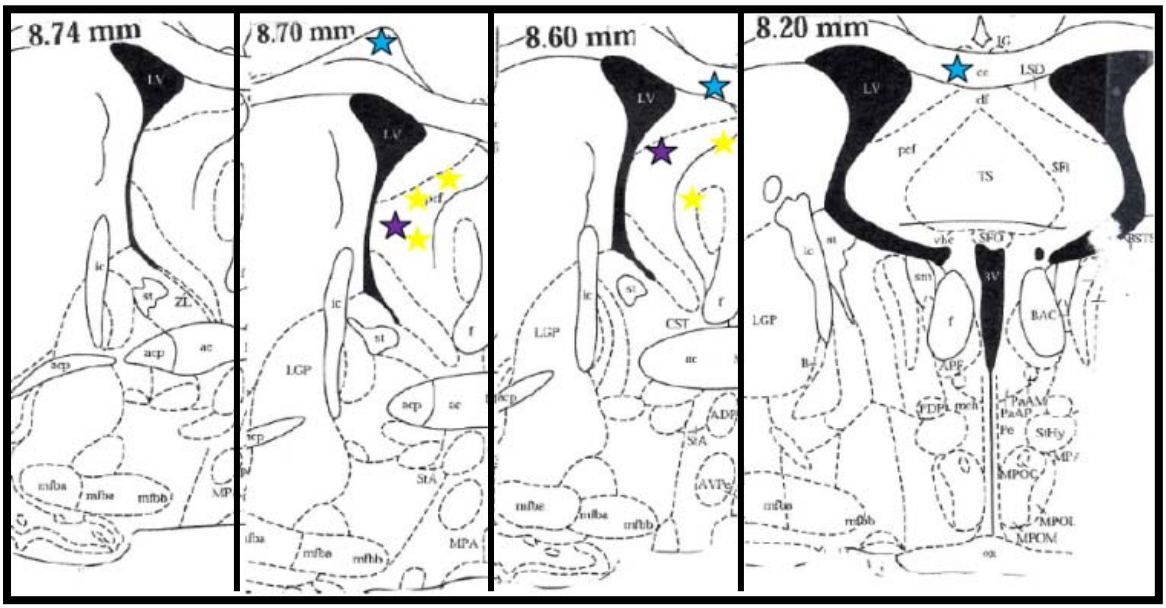

Figure 31: Mapping of injection sites for 1.0 µg of carbachol injected into the LS. The stars are colour coded for the number of 22-kHz USVs. Blue stars were able to initiate 0 22-kHz USVs, yellow stars were able to initiate 100-200 22-kHz USVs, and purple stars were able to initiate 201-300 22-kHz USVs. The numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. Selected abbreviations see Figure 12b.

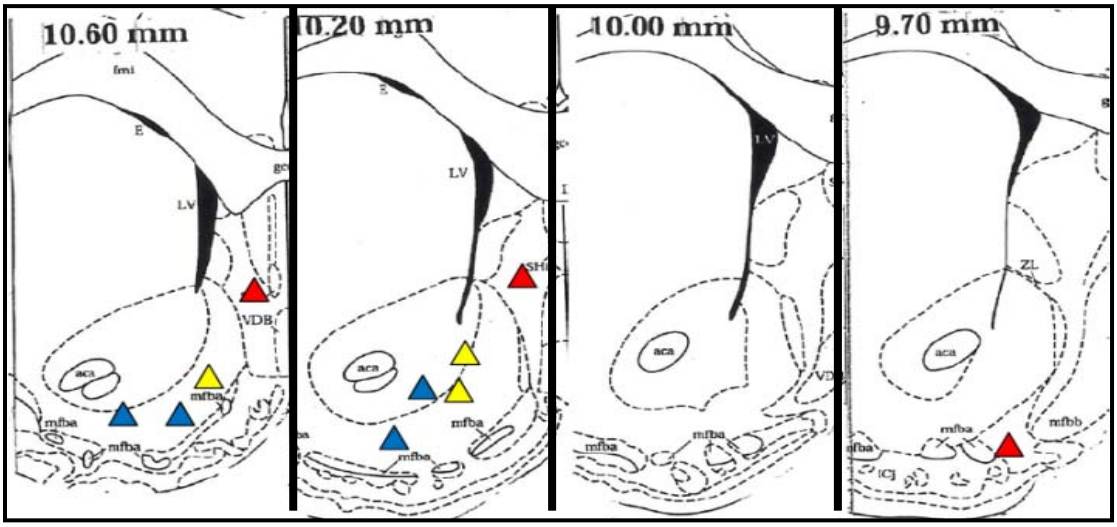

Figure 32: Mapping of injection sites for 3.0 µg of apomorphine injected into the shell of the nucleus accumbens. The localization triangles are colour coded for the magnitude of the response. Red circles were able to induce 0 50-kHz USVs, blue triangles were able to elicit 1-10 50-kHz USVs and yellow triangles were able to elicit 10-20 50-kHz USVs. The numbers at the top of the diagrams represent frontal planes in mm from the stereotaxic zero. For selected abbreviations see Figure 1.

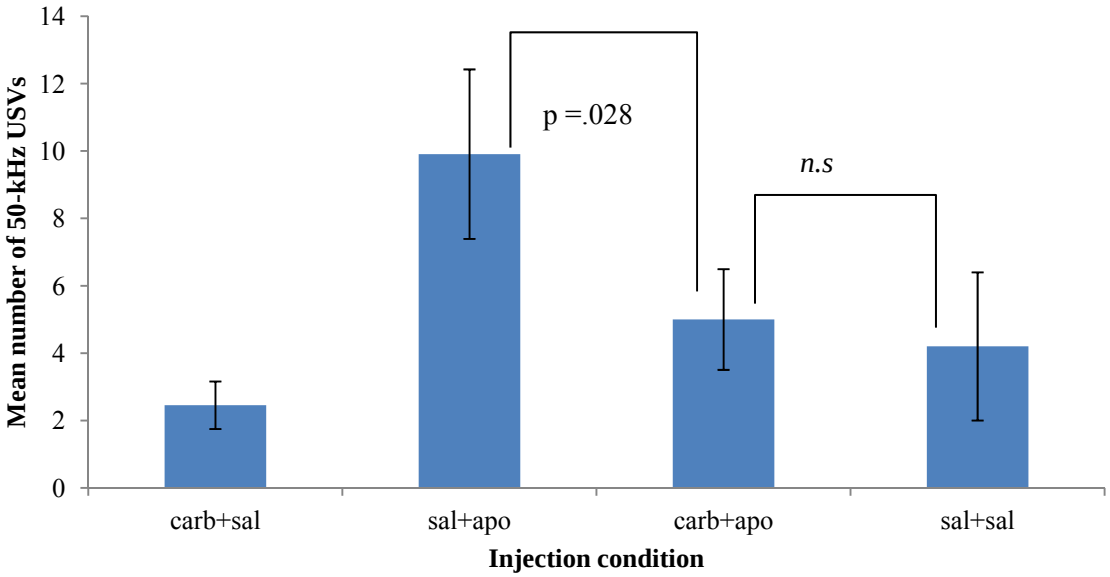

Figure 33: Mean number of 50-kHz USVs (± S.E.M.) recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean number of 50-kHz USVs emitted. Injection of carbachol (carb) into the LS, one minute before an injection of apomorphine (apo) into the medial nucleus accumbens, was able to significantly reduce the mean number of 50-kHz USVs. Injection conditions were as follows: carb+sal – carbachol was injected into the LS and saline was injected into the medial shell of the nucleus accumbens; sal+apo – saline was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the LS and saline was injected into the medial shell of the nucleus accumbens.

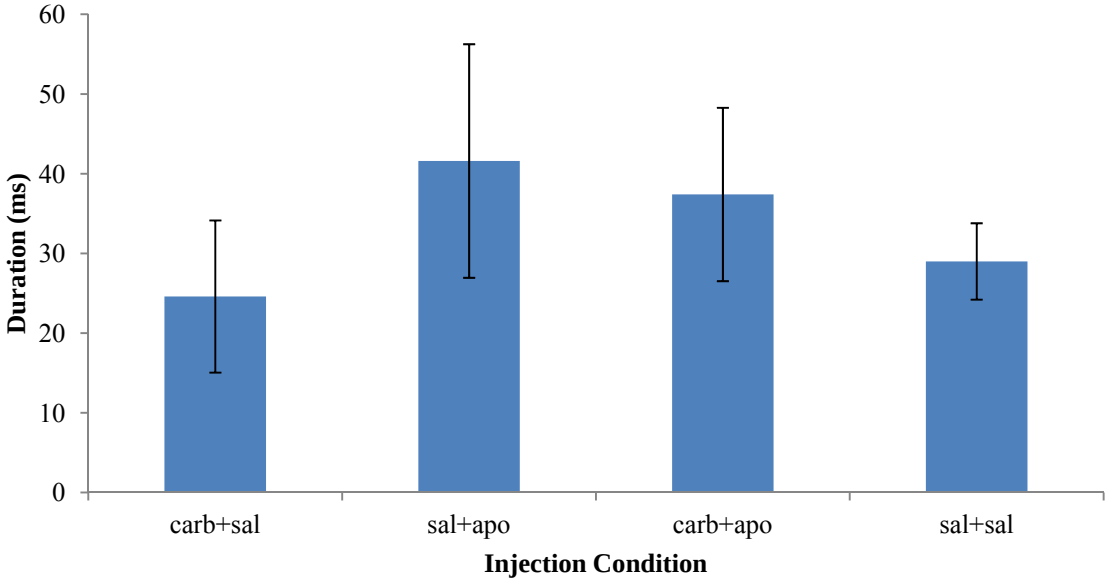

Figure 34: Mean duration (± S.E.M.) of the same vocalizations analyzed in figure 33. The x-axis represents the various injection conditions and the y-axis represents the mean duration (ms) of 50-kHz USVs emitted. Injection of apomorphine into the medial shell of the nucleus accumbens, one minute after an injection of carbachol into the LS, was unable to significantly change the duration of 50-kHz USVs USVs. Injection conditions were as follows Injection conditions were as follows: carb+sal – carbachol was injected into the LS and saline was injected into the medial shell of the nucleus accumbens; sal+apo – saline was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the LS and saline was injected into the medial shell of the nucleus accumbens.

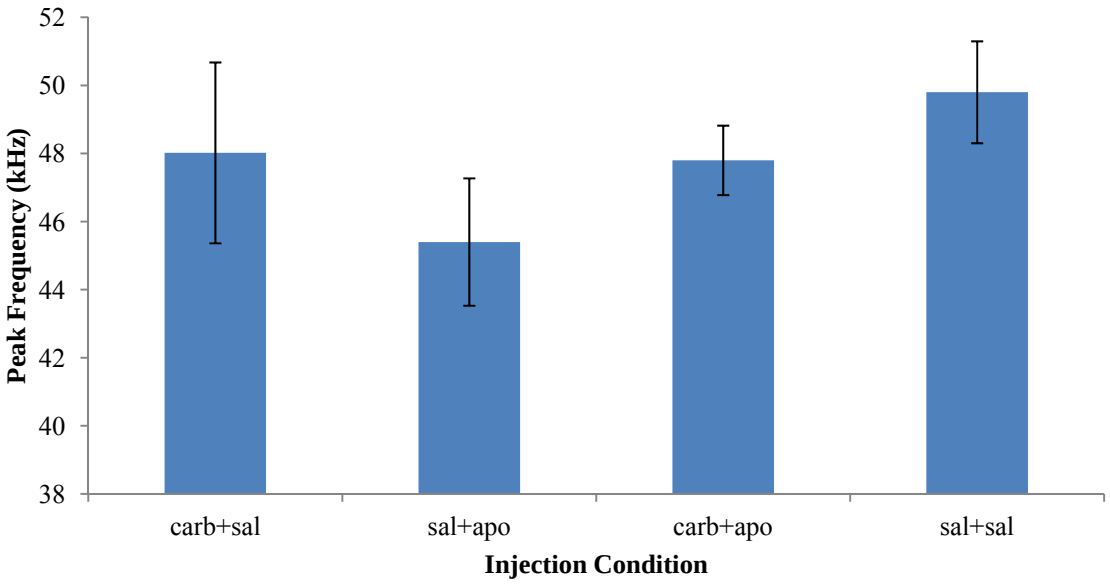

Figure 35: Mean peak frequency (± S.E.M.) of 50-kHz USVs recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean peak frequency of 50-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute after an injection of carbachol (carb) into the LS, was unable to significantly change the mean peak frequency of 50-kHz USVs USVs. Injection conditions were as follows Injection conditions were as follows: carb+sal – carbachol was injected into the LS and saline was injected into the medial shell of the nucleus accumbens; sal+apo – saline was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the LS and saline was injected into the medial shell of the nucleus accumbens.

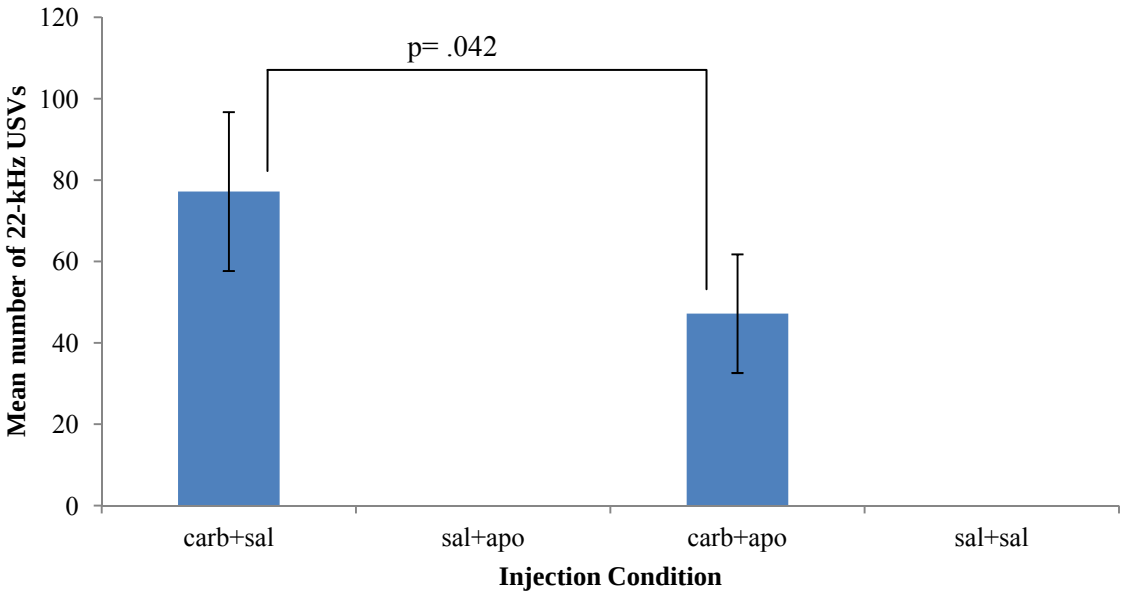

Figure 36: Mean number of 22-kHz USVs (± S.E.M.) recorded under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean number of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute after an injection of carbachol (carb) into the LS, was able to significantly reduce the mean number of recorded 22-kHz USVs. Injection conditions were as follows Injection conditions were as follows: carb+sal – carbachol was injected into the LS and saline was injected into the medial shell of the nucleus accumbens; sal+apo – saline was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the LS and saline was injected into the medial shell of the nucleus accumbens.

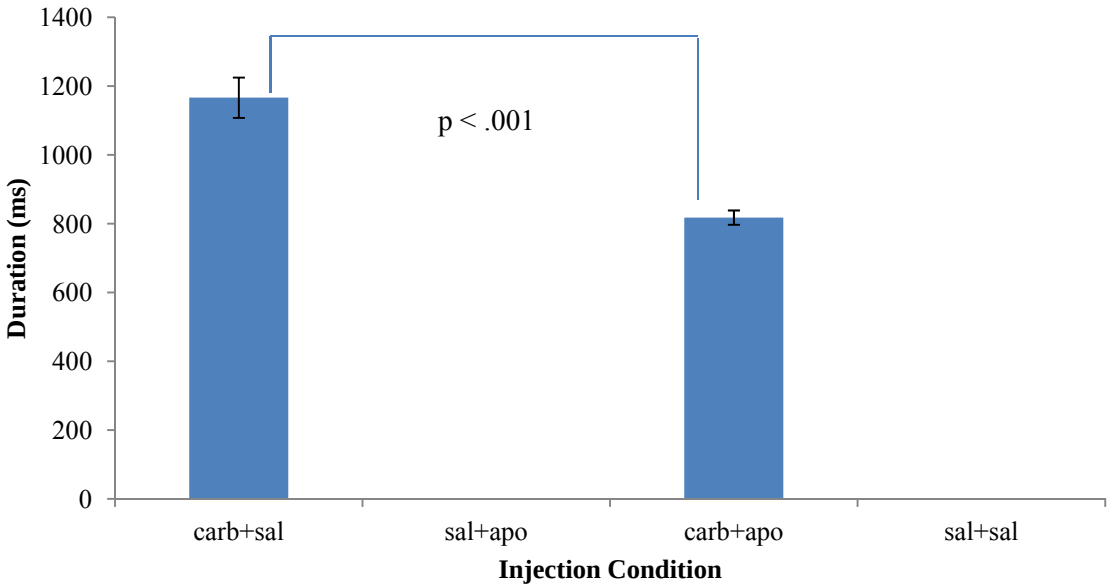

Figure 37: Mean duration (± S.E.M.) of the same vocalizations analyzed in figure 36. The x-axis represents the various injection conditions and the y-axis represents the mean duration of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute after an injection of carbachol (carb) into the LS, was able to significantly alter the duration of the 22-kHz USVs. Injection conditions were as follows Injection conditions were as follows: carb+sal – carbachol was injected into the LS and saline was injected into the medial shell of the nucleus accumbens; sal+apo – saline was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the LS and saline was injected into the medial shell of the nucleus accumbens.

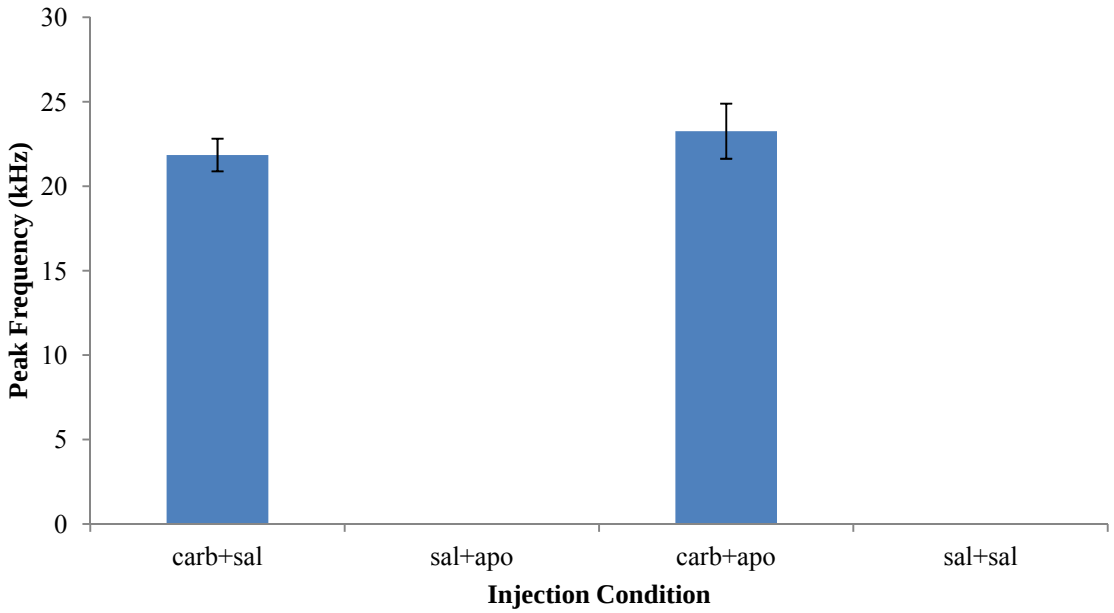

Figure 38: Mean peak frequency (± S.E.M.) of 22-kHz USVs under different injection conditions during a five minute recording session. The x-axis represents the various injection conditions and the y-axis represents the mean peak frequency of 22-kHz USVs emitted. Injection of apomorphine (apo) into the medial shell of the nucleus accumbens, one minute after an injection of carbachol (carb) into the LS, was unable to alter the PF of the 22-kHz USVs. Injection conditions were as follows Injection conditions were as follows: carb+sal – carbachol was injected into the LS and saline was injected into the medial shell o the nucleus accumbens; sal-apo – saline was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; carb+apo – carbachol was injected into the LS and apomorphine was injected into the medial shell of the nucleus accumbens; sal+sal – saline was injected into the LS and saline was injected into the medial shell of the nucleus accumbens.

Discussion

The goal of this thesis was to provide evidence of the functional relationship between the ascending mesolimbic dopamine system and the ascending mesolimbic cholinergic system and to describe the relationship between the two systems. Four predictions were formulated from the hypothesis. The first prediction stated that apomorphine will dose-dependently increase the number of 50-kHz USVs since this category of USVs are dependent on dopamine transmission in the shell of the accumbens, the 2nd and 3rd prediction focused on the reduction in aversive or appetitive USVs upon activation of the opposing emotional system, and the 4th prediction stated the parameters of the USVs would not change because they are instrumental in conveying the message.

Evaluating the first prediction: Apomorphine's ability to increase the emission of 50-kHz USVs

The doses of apomorphine that were used to establish a relationship between apomorphine dose and production of 50-kHz USVs were 0.5 µg, 1.0 µg, 1.75 µg, 2.5 µg and 3.0 µg. The high doses of apomorphine, but not the low doses, were able to increase the production of 50-kHz USVs which is consistent with the biphasic pharmacological profile of apomorphine action (Di Chiara et al., 1976). Low doses of apomorphine have been reported to inhibit dopamine cell firing, decrease dopamine metabolism, inhibit dopamine release, decrease locomotor activity in rats and increase yawning in rats (Lynch, 1991). High doses of apomorphine have been reported to cause the opposite physiological and behavioural changes compared to low doses of apomorphine including, but not limited to, increased dopamine metabolism, increased locomotor activity, increased gnawing compulsion, and increased dopamine cell firing (Lynch, 1991). The dose-response relationship of apomorphine induced 50-

kHz USVs is consistent with the literature describing apomorphine's pharmacological behaviour. Injection of 0.5 μg and 1.0 μg caused the lowest mean number of 50-kHz USVs while 3.0 μg caused the largest increase in emitted 50-kHz USVs. However, apomorphine was only able to increase the number of flat 50-kHz USVs and did not significantly increase the number of FM 50-kHz USVs.

Emerging evidence suggests the FM 50-kHz USVs are dependent upon activation of D_3 receptors. Brudzynski and colleagues reported that injection of low doses of quinpirole, a selective D_2/D_3 agonist, was able to increase the number of FM vocalizations over amphetamine, a drug that increases synaptic concentration of dopamine. Increases in FM 50-kHz USVs by quinpirole were selectively attenuated by pretreatment of the shell of the nucleus accumbens with U-991941, a selective D_3 receptor antagonist (Brudzynski, et al, 2012). Although the precise mechanism that causes an increase in FM 50-kHz USVs after activation of D_3 receptors is unknown, it does explain why there was a failure of apomorphine to increase FM 50-kHz USVs since apomorphine has been reported to be predominantly a D_1/D_2 receptor agonist.

Evaluating predictions 2 and 3: The relationship between the two emotional systems is antagonistic in nature

Activation of the mesolimbic dopamine system prior to activation of the ascending mesolimbic cholinergic system was able to reduce the number of 22-kHz USVs. Conversely, activation of the ascending mesolimbic cholinergic system before activation of the mesolimbic dopaminergic system was able to decrease 50-kHz USVs. This decrease in both appetitive and aversive USVs by prior activation of the opposing system provides evidence that the two systems interact with each other and the nature of their interaction is antagonistic. This answers the

central question of the thesis and satisfies the main goal. However, a problem with the central hypothesis does arise since the mechanism of interaction predicts the absence of vocalization during the activation of both emotional systems, since they are antagonistic to each other. This was not demonstrated. Both 50-kHz and 22-kHz USVs were recorded after activation of the opposing emotional system. This raises the possibility that the two systems are not directly antagonistic along the neuraxis of the rat, but rather there exist brain nuclei that are able to toggle the activation of one emotional system over another.

If such brain nuclei exist that help mediate the activation of one system over another then these brain nuclei should be activated during aversive or appetitive conditions which would increase the release of acetylcholine into the terminal fields of the medial cholinoceptive vocalization strip or increase the release of dopamine into the shell of the nucleus accumbens, respectively. One potential nucleus that satisfies such criteria is the LTDg.

LTDg-VTA connection: Increasing dopamine within the nucleus accumbens during reward

Tracing studies coupling lectin tracers with immunocytochemical detection has revealed that the LTDg projects heavily to both medial and ventral sections of the VTA (Cornwall et al, 1990) using glutamate, acetylcholine and GABA releasing neurons. Although the exact physiology and function of the LTDg-VTA projection is complex, it has been reported that both glutamate and acetylcholine projections onto dopamine containing neurons in the VTA are able to indirectly increase the release of dopamine within the nucleus accumbens (Forester and Blaha, 2000; Forester et al, 2001). Using chronoamperometry, Forester and Blaha (2000) reported that electrical stimulation of the LTDg caused an increase in dopamine oxidation current measured in the shell of the nucleus accumbens. The increase in dopamine oxidation current is dependent

upon both glutamatergic and cholinergic from the LTDg since intra-VTA infusion of mecamylamine, a nicotinic antagonist and kynurenate, a glutamatergic antagonist, blocked the increase in dopamine oxidation current in response to LTDg stimulation. It has also been reported that the m5 muscarinic receptor also contributes to the prolonged release of dopamine within the nucleus accumbens during reward (Forester et al, 2001). Yeomans and colleagues (2001) reported the increases in dopamine release into the nucleus accumbens after rewarding electrical stimulation of the lateral hypothalamus, a condition that has been reported to increase 50-kHz USVs in rats, can be decreased in rats with truncated m5 receptors being expressed on dopamine cell bodies in the VTA (Yeomans et al, 2001) of mice. Cholinergic innervation of dopamine neurons that expressed the m5 receptor was provided by the LTDg (Yeomans et al, 2001). Innervation of the VTA by the LTDg is involved with the release of dopamine into the nucleus accumbens, but it is also involved in changing the firing activity of dopamine neurons from irregular to phasic.

Dopamine neurons within the VTA display two types of firing properties. Under basal situations dopamine cell bodies fire in an irregular and uncoordinated manner, but under conditions that are considered to be rewarding, the dopamine cells change their firing patterns from irregular to phasic (Mileykovskiy and Morales, 2011; Mirenowicz and Schultaz, 1996). This phasic firing pattern of dopamine is able to increase the release of dopamine through the mesolimbic dopamine system, especially the shell of the nucleus accumbens (Ikemoto, 2007; Matsumoto and Hikosaka, 2007; Matsumoto and Hikosaka, 2000). This change in dopamine firing activity from irregular to phasic does partially depends on the glutamatergic input from the LTDg. Support of this relationship is demonstrated by intra-VTA injections of AMPA antagonists, such as (±)-2-amino-5-phosphonopentanoic acid, which can block the ability of

dopamine neurons to transition from irregular to rhythmic firing patterns in response to LTDg stimulation (Lodge and Grace, 2006). The LTDg-VTA connection is an important connection that allows for the proper functioning of VTA dopamine neurons in response to rewarding situations (Yeomans et al, 2001; Lodge and Grace, 2006). No studies have stimulated the LTDg pharmacologically and investigated the resulting 50-kHz USVs vocalizations in rats.

The glutamatergic and cholinergic projection from the LTDg-VTA seems to be excitatory in both cases. However, electrophysiological recordings of dopamine neurons in aversive contexts show a sharp depression in dopamine firing patterns (Mileykovskiy and Morales, 2011; Mirenowicz and Schultaz, 1996). Thus activation of the LTDg under aversive contexts probably uses an indirect connection to inhibit the firing for dopamine neurons within the VTA. The lateral habenula (LH), a nucleus located within the epithalamus may be one such nucleus that relays negative values to the dopamine cells within the VTA.

The habenula and the dorsal diencephalic conduction system

The LTDg has a strong reciprocal relationship with the LH (Cornwall et al, 1990) via the dorsal diencephalic condition system (DDC; Sutherland, 1982). The DDC system comprises the afferent system, the stria medullaris (sm), and its efferent system, the fasciculius retroflexus (fr; Brinschqitz et al, 2010). This DDC system is one of the main fiber systems that are used for communication between forebrain nuclei and catacholaminergic cell bodies located within the mesencephalon; this includes the VTA and the substantia nigra pars compacta. The medial root of the fr is composed of fibers from the medial habenula (MH) that terminate mainly on neurons localized within the interpeduncular nucleus, a prominent motor nucleus of the basal ganglia, in the rat (Hikosaka et al, 2008). The LH however is distinct from the MH in its fibers termination point. The LH does not primarily terminate on TH+ dopamine neurons in the VTA but rather

terminates with a large number of glutamatergic fibers on GAD positive neurons located in the rostromedial tegmental nucleus. (Hikosaka et al, 2008; Jt and Shperard, 2007; Matsumoto and Hikosaka, 2007). The rostromedial tegmental nucleus contains GABAergic neurons that project heavily to dopamine neurons located within the VTA. Essentially this connection allows the excitation of the LH neurons to excite GABA projection neurons in the rostromedial tegmental nucleus to release GABA, an inhibitory neurotransmitter onto dopamine neurons in the VTA causing them to hyperpolarize and decrease the release of dopamine along the mesolimbic dopamine system (Hong et al, 2011). This functionally antagonistic relationship is highlighted by metabolic, lesion and electrophysiological investigations.

LH to VTA projection system: An antagonistic relationship

Metabolic studies have suggested an antagonistic relationship between the LH and the VTA. Systemic injections of dopamine agonists, such as R-(-)-apomorphine or *d*-amphetamine decrease 2-deoxyglucose (a measure of neuronal metabolic activity) utilization in the LH (Wechsler et al, 1979). Likewise injection of dopamine antagonists, such as haloperidol, increases the metabolic activity of the LH while decreasing the activity of the VTA and substantia niagra (McCulloch et al, 1980). The antagonistic relationship between the LH and the VTA was further supported by lesioning studies.

Injection of the dopaminergic neurotoxin, 6-hydroxydopamine, into the mesolimbic dopamine system, can increase the metabolic activity of the LH. Injections of TTX, a neurotoxin causing the cessation of neural activity, into the stria medullaris causes large increases in dopamine metabolism in the prefrontal cortex, olfactory tubercle and nucleus accumbens, as measured by levels of homovanilic acid (Nishikawa et al, 1986). Dopaminergic innervation of the prefrontal cortex, olfactory tubercle and nucleus accumbens comes from dopaminergic cell

bodies within the VTA. Further support that the LH mediates the decreased dopamine activity through the DDC comes from electrophysiological experiments.

If the dopamine neurons located within the VTA encode a reward or positive emotional state, and neurons in the LH encode a negative emotional state, then rewards should increase the activity of dopamine neurons in the VTA while inhibiting neurons in the LH. Conversely, noxious stimuli or the absence of reward should have opposing effects on the VTA and LH neuron activity causing LH neurons to depolarize (become active) and VTA neurons to hyperpolarize. Such a relationship was reported in monkeys by Matsumoto and Hikosaka (2007). Monkeys that were trained to respond to two types of visual cues, one visual cue that predicts a reward and one visual cue that predicts the absence of a reward, a paradigm that has been used to produce 50-kHz and 22-kHz USVs, respectively (Burgdorf, 2000). The results reported by Matsumoto and Hikosaka (2007) showed that the LH and the VTA have opposing firing activity. Visual cues that signal the presentation of a reward (juice) cause excitation in VTA neurons and an inhibition of LH neurons while visual cues that represent a negative, or punishing outcome (a jet of air aimed at the palm of the monkey) show excitation of the LH and inhibition of the VTA neurons (Matsumoto and Hikosaka, 2007). Similarly, Gao and colleagues (1996) reported that peripheral noxious stimulus (identified as a shock applied to the tail of the rat) increased the firing rate of LH neurons and inhibited substantia nigra pars compacta neurons (Gao et al, 1996). These results are congruent with Christoph and colleagues (1986) who reported stimulation of the LH in the rat causes an attenuation of 91% of dopamine-identified neurons within the VTA and 85% of dopamine-identified neurons within the substantia nigra pars compacta (Christoph et al, 1986).

Despite the results suggesting a highly antagonistic relationship between the LH and the VTA, few studies have been done describing the relationship between the LTDg and the LH. Indirect Fos-immunoreactivity experiments investigating brain nuclei responsible for processing aversive stimuli show a consistent increase in Fos immunoreactive protein in both these brain structures (Martinez et al, 2002; Singewald et al, 2003). This is consistent with anatomical mapping studies showing that the LTDg and the LH have a strong reciprocal relationship (Herkenham and Nauta, 1977). Despite this anatomical connection, and the involvement of these nuclei during punishing and negative emotional-arousing situations, their electrophysiological relationship remains to be investigated.

Indirect evidence of a functional relationship between the LH and the LTDg can be observed using Fos-immunoreactivity. Both the LH and the LTDg show increased activity during the production of 22-kHz USVs or during the playback of 22-kHz USVs. Brudzynski and colleagues showed doubled labeled cholinergic cells in the LTDg in response to carbachol induced 22-kHz USVs from the AH-MPO (Brudzynski, 2011). Playback studies using 22-kHz USVs show increased Fos-immunoreactivity within the main input nucleus of the habenula complex (Beckett et al, 1997) and LTDg (Sadananda et al, 2008) supporting the hypothesis of a functional relationship between the LTDg and the LH during aversive situations.

LTDg-LH-VTA connection during negative emotional processing

If a functional relationship between the LTDg-LH-VTA exists, it would allow the ascending mesolimbic cholinergic system to use the LH to shut down activity of the ascending mesolimbic dopamine system during aversive situations. Such a relationship does explain why activation of the mesolimbic cholinergic system, by carbachol injections into the LH or the AH-MPO, was able to decrease the number of apomorphine emitted 50-kHz USVs. Further

electrophysiological, immunohistochemical and behavioural investigations will need to be conducted to establish that such a relationship exists in the rat during the production of aversive USVs.

VTA-LH-LTDg connection during positive emotional processing

Anticipation of rewards increases the activity of dopamine neurons within the VTA (Mirenowicz and Schultz, 1996), increase dopamine efflux in the shell of the nucleus accumbens (Knuston et al, 2001), and inhibits the firing of LH neurons (Mirenowicz and Schultz, 1996). Electrophysiological experiments show that the connections between the VTA and the LH are antagonistic since electrical stimulation of the VTA is able to cause a current-dependent inhibition of 90% of aversion-related LH neurons (Shen et al, 2012). Although the authors did not do any pharmacological analysis of the transmitter's involved VTA-LH connection, in situ hybridization studies have reported D_2 mRNA localized within the LH (Brouwer et al, 1992). Since D_2 receptors are linked to increases in potassium-related currents (Vallar & Meldolesi, 1989), which suppresses neuronal activity of a neuron by hypopolarizing the cell, this could explain the reported inhibition of LH neurons by VTA activity. Another mechanism could be reward related signals projected from the edge cells onto LH neurons, but this functional link has only been described in primates (Hong & Hikosaka, 2008). Since the edge cells are localized within the internal globus pallidus in primates, a similar function connection would have to exist within the entopeduncular nucleus of the rat, since this is the homolog to the internal division of the globus pallidus in primates. Such connects have been reported (Kooey and Carter, 1981) but a functional relationship has yet to be established.

To explain the decreased production of carbachol-induced 22-kHz USVs by prior activation of the mesolimbic dopamine system, a direct antagonist relationship would also have

to exist between the LH and the LTDg whereby inhibition of the LH would cause an inhibition of neurons within the LTDg. Such a functional relationship has not been reported. Further investigation of the LH during 50- and 22-kHz USVs would be needed to confirm the operation of such a circuit between the LH and LTDg. Although the central question of the thesis was answered, the mechanisms remain elusive.

Evaluation of Question 4

Mean call duration and mean peak-frequency values were used to characterize the acoustic features recorded USVs. The mean value of all obtained 50-kHz USVs in this thesis fell within acceptable ranges of 50-kHz USVs reported within the literature (Brudzynski, 2009, Burgdorf, 2000). Peak-frequency of the vocalizations fell between 38-90 kHz and the duration of the recorded 50-kHz fell between 10-80 ms. Under no experimental conditions did the peak frequency or the duration of 50-kHz USVs change. This suggests that duration and peak-frequency of 50-kHz USVs may be needed to convey appetitive information. This was consistent with the prediction.

The mean values of all obtained 22-kHz USVs in this thesis also fell within the reported ranges of 22-kHz USVs (Bihari et al, 2003). These recorded USVs had a peak frequency of 19-29 kHz and duration between 100 – 3000 ms. As reported in the results section, the duration of 22-kHz USVs under all conditions were significantly reduced with activation of the mesolimbic dopamine system. This was not consistent with the fourth prediction. The consistent decrease in the duration of recorded 22-kHz USVs may signal the degree of anxiety in the sender; the shorter the 22-kHz USVs the less anxiety the animal is conveying. Playback studies using 22-kHz USVs of varying duration will be able to support or refute such a conclusion.

Localization of injection sites

Localization of injections for the dose-response study, as well as the double-injection groups for the nucleus accumbens, were found between 10.6 mm – 9.7 mm, laterally from 0.7 – 1.3 mm from the midline making the resulting injections span across the core and shell of the nucleus accumbens. Analysis of the magnitude of pharmacologically-induced 50-kHz USVs in the nucleus accumbens showed that the highest sensitivity to apomorphine was found within the medial shell of the nucleus accumbens and decreased laterally across the shell. The core of the nucleus accumbens, which is related to the motor system (Zahm & Brog, 1992), produced substantially lower 50-kHz USVs when compared to shell. The ability of the core to produce a low-level of 50-kHz USVs is likely due to diffusion of the drug out of the core and onto the medial border of the shell. Injection into pallidum, fiber tracts, or ventricles failed to produce any 50-kHz USVs.

Localization of injections for the AH-MPO and the LS were in accordance with injection sites reported in the literature to produce 22-kHz USVs (Bihari et al, 2003; Brudzynski, 2011). Injections into the ventricles, or fiber tracts were unable to produce any 22-kHz USVs.

Future research

Future research stems from three perspectives. The first is the pharmacological action of carbachol which is a broad spectrum muscarinic agonist with some affinity for nicotinic receptors. Since muscarinic and nicotinic receptors are expressed within the AH-MPO and the LS, it would be of importance to tease out whether selective muscarinic or nicotic or both receptors are involved in the expression of aversive states in rats. The second perspective is the functional relationship between the LTDg and the LH. It would be interesting to record the

responses of the LH neurons to application of glutamate into the LTDg, an injection condition that is known to produce the emission of 22-kHz USVs, this would provide direct evidence of the relationship between these two structures during the production of 22-kHz USVs. The third perspective is viewing the LTDg as a station that can toggle mesencephalic nuclei for the initiation of different emotional states. Since the LTDg is a primarily cholinergic structure, it would have to contain two or more distinct populations of cholinergic neurons. One group of these cholinergic cell bodies would interact with the ascending mesolimbic dopamine system during appetitive circumstances. The second cluster of cholinergic neurons would be responsible for releasing acetylcholine into the terminal fields of the mesolimbic cholinergic system and subsequently activating the LH to inhibit the dopamine containing cell bodies within the VTA. The activity of the two different clusters of cholinergic cells would be toggled by activation of different GABAergic interneurons that receive excitatory glutamatergic input from multiple limbic brain structures along the neuraxis. Interestingly, the LTDg expresses mRNA for glutamic acid decarboxylase (GAD), a cellular marker for GABAergic releasing neurons, in about 5% of cells (Wang & Morales, 2009). It would be interesting if this small cluster of GABAergic cells functioned in the a similar, yet opposing, manner as the small cluster of GABAergic cells located within the rostromedial tegmental nucleus that are activated in negative emotional situations to inhibit dopamine cell bodies within the VTA. This would provide a mechanism to inhibit the LTDg during appetitive states by activation of these GABAergic cells, which would inhibit acetylcholine release from the LTDg into the medial cholinoceptive vocalization strip.

Conclusions

The central question that this thesis focused on was if the systems governing the expression of emotional states in rats interact, and what is the relationship of this interaction.

Activation of the mesolimbic dopamine system, prior to activation of the ascending mesolimbic cholinergic system, was able to decrease the number of aversive 22-kHz USVs while activation of the mesolimbic cholinergic system, prior to activation of the ascending mesolimbic dopamine system, was able to decrease the number of 50-kHz USVs. The decrease in 22-kHz and 50-kHz USVs indexed a selective decrease in an appetitive state or an aversive state. The central question was answered. It can be concluded that the neural systems responsible for aversive and appetitive USVs interact, and that the nature of this interaction is antagonistic.

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